Advanced Therapy Medicinal Products: Clinical, Non-clinical, and Quality Considerations

14

Enrico Mastrobattista, Erik Doevendans, Niek P. van Til, Vera Kemp, Jeroen de Vrij, and Karin Hoogendoorn

Introduction

The most recent branch of the biotechnology revolution in medicine consists of gene and cell therapy medicinal products and tissue-engineered products. These are collectively called advanced therapies in the US and advanced therapy medicinal products (ATMPs) in the EU. The use of living cells, tissues, or organs in medical practice is not novel. The first successful kidney transplantation in a human took place in 1954, and human hematopoietic stem cell transplantation from a healthy donor to a cancer patient in 1959; the latter is now a routine clinical procedure for bone marrow regeneration. Cell therapies, including stem cell therapies, were not further explored until the early 1990s when the therapeutic relevance of mesenchymal stromal cells (MSCs) was considered for the regeneration of skeletal tissue and later for broader therapeutic use. Furthermore, the development of efficient gene transfer vectors to genetically modify cells ex vivo or directly in vivo in the 1980s and 1990s further boosted the development of therapies based on genetically corrected or augmented cells. Since the turn of the millennium, there has been a steady increase in the number of clinical studies, with a growing number of target indications. Particularly for the treatment of diseases and tissue/organ defects for which traditional therapies and medicinal products have not always provided positive benefit/risk outcomes, such as multiple sclerosis, Parkinson's disease, cancer, and muscular dystrophy, advanced therapies hold high expectations. The inherent complexity of these products poses unique challenges compared to other therapeutics. The manufacture of gene transfer vectors or "living" materials (i.e., cells and tissues) comes with great challenges in terms of consistency and process and product characterization. Such challenges are analogous, in many ways, to those faced in the past when the first recombinant protein biopharmaceutical products were being developed and regulated. Bringing advanced therapies to market at an acceptable cost, benefit/risk ratio, and quality has proven extremely difficult for certain products.

This chapter discusses the current status and unique aspects of ATMPs. We explain the differences between traditional cell or tissue transplantation versus advanced therapies based on (genetically manipulated) somatic cells or tissue-engineered products. Then, we discuss in detail the various cell technologies and technologies for gene therapy and provide information on the manufacturing of advanced therapies. Finally, the regulatory aspects of ATMPs will be briefly highlighted.

ATMPs: Definitions, Classifications, and Modes of Action

Globally, different names and definitions for gene- and cell-based products are used in different jurisdictions. For example, in the EU, such a product is called an advanced therapy medicinal product (ATMP), whereas the term "advanced therapy" is used in the USA. Minor differences in the defini-

E. Mastrobattista (✉) · E. Doevendans
Pharmaceutics Division, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands
e-mail: E.Mastrobattista@uu.nl; E.Doevendans@uu.nl

N. P. van Til
Department of Child Neurology, Amsterdam Leukodystrophy Center, Emma Children's Hospital, Amsterdam University Medical Centers, VU University, and Neuroscience, Cellular & Molecular Mechanisms, Amsterdam, The Netherlands
e-mail: N.P.vanTil@amsterdamumc.nl

V. Kemp
Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, The Netherlands
e-mail: V.Kemp@LUMC.nl

J. de Vrij
Department of Neurosurgery, Brain Tumor Center, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands
e-mail: J.deVrij@ErasmusMC.nl

K. Hoogendoorn
Hospital Pharmacy, Interdivisonal GMP Facility, Leiden University Medical Center, Leiden, The Netherlands

D. J. A. Crommelin et al. (eds.), *Pharmaceutical Biotechnology*, https://doi.org/10.1007/978-3-031-30023-3_14

tion of (sub-)classes between the two jurisdictions exist, and importantly, in the US, human cells and tissues may also be regulated as devices (similarly to the combined ATMPs in Europe). Furthermore, because of the minor differences in definitions applied in the US and EU, hematopoietic stem cell (HSC) transplantation for the treatment of malignant blood disorders is an example of a product that would be classified as an advanced therapy in the US but would fall outside the scope of ATMPs in the EU (see section "Adult Stem Cells Used as Transplant Product"; Fig. 14.5).

Transplantation or Advanced Therapy?

Advanced therapies, when applied to humans, are considered biological medicinal products, meaning they are typically subject to either or both of the following regulatory regimes: public health legislation and pharmaceutical legislation. However, some clinical interventions for cell- and tissue-based advanced therapies are not considered "medicinal"; these products are subject to public health legislation only. These therapies are often called "cell and tissue transplant products" or "cell and tissue transplantations" and have to meet all of the following criteria (see also Fig. 14.1):

1. A cell or tissue, which is not substantially manipulated. Table 14.1 provides guidance on the definition of substantial and nonsubstantial manipulations.
2. Cells/tissues are used for the same essential function in the donor and recipients (sometimes called "homologous use").
3. It is not combined with a medical device or active implantable medical device.

Cell and tissue transplant products require no clinical trials and no marketing authorization (MA) prior to commercial availability but public health legislations apply.

However, if cells or tissue are substantially manipulated, it fulfills the criteria for an advanced therapy medicinal product (ATMP) and, as such, will be regulated as a medicine, meaning the development must comply with medicines regulations.

ATMPs can be subdivided into somatic cell therapy medicinal products (SCTMP), consisting of somatic cells that have been expanded and/or manipulated ex vivo before being administered back into the patient; gene therapy medicinal products (GTMP), consisting of vectors for transfer of materials that can genetically modify cells ex vivo or in vivo; tissue-engineered products (TEP), involving cells or multiple cell types growing on a scaffold to form a 3D tissue culture; and cells or tissues that are being combined with a medical device (combined ATMP) (Fig. 14.1 and Table 14.2).

Gene or Cell Therapy Medicinal Product?

There is considerable overlap between somatic cell therapy medicinal products (SCTMP) and gene therapy medicinal

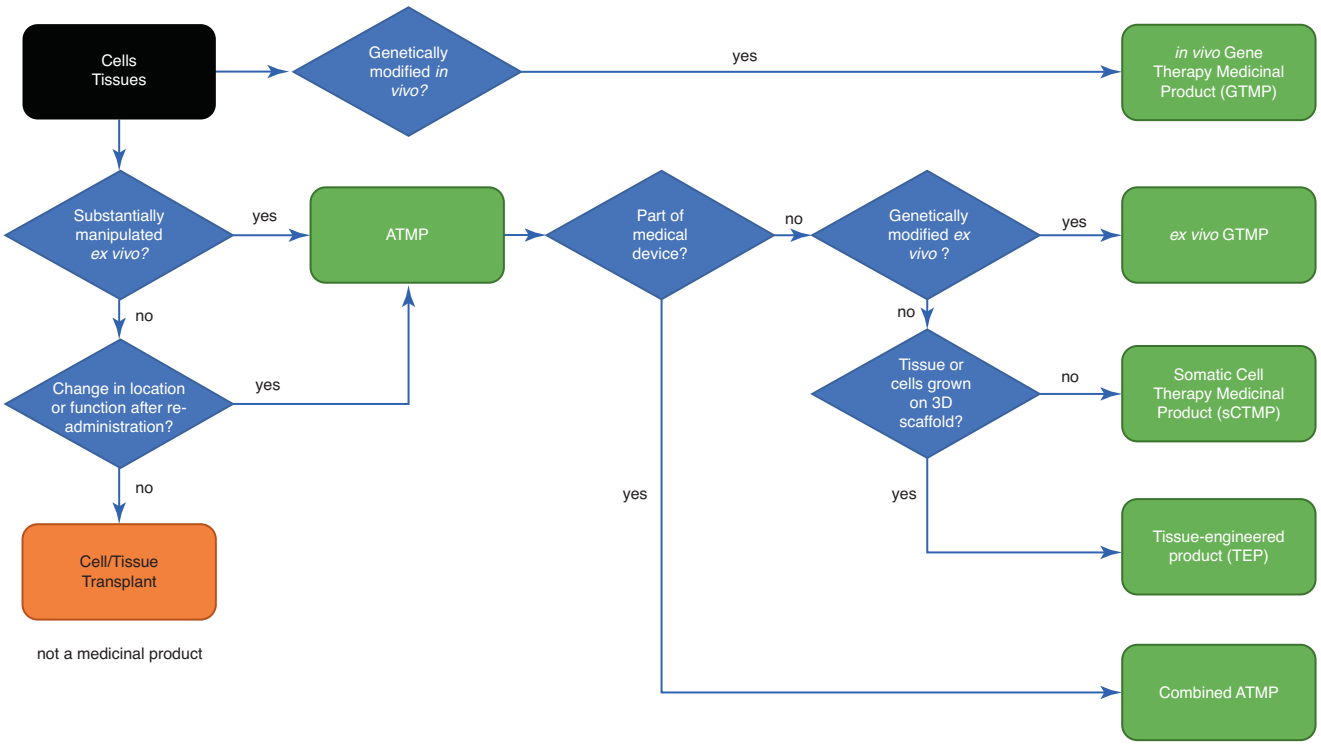

Fig. 14.1 Decision tree for categorizing ATMPs. If cells or tissues are not substantially manipulated and are used for the same essential function in donor and recipient (homologous use), the therapy is not considered an ATMP but categorized as a cell or tissue transplant, for which different regulations apply. If cells are substantially manipulated or genetically modified, these products are considered ATMPs. For definitions of substantial cell manipulation, see Table 14.1

Table 14.1 Substantial and nonsubstantial manipulations of cells or tissues

Substantial manipulation	Non-substantial manipulation
Specific manipulations considered substantial are: 1. Cell expansion (culture; ex vivo) 2. Differentiation and/or activation with growth factors 3. Ex vivo genetic modifications of cells (e.g., with viral vector)	Specific manipulations *not* considered substantial: 1. Cutting 2. Grinding 3. Shaping 4. Centrifugation 5. Soaking in antibiotic or antimicrobial solutions 6. Sterilization 7. Irradiation 8. Cell separation, concentration or purification 9. Filtering 10. Lyophilization 11. Freezing 12. Cryopreservation 13. Vitrification

products (GTMP). If a cell is isolated from the body and substantially manipulated ex vivo before readministration, it is considered a cell therapy medicinal product. Suppose (part of) this substantial manipulation consists of genetic modification; the product of this manipulation is considered as an (ex vivo) gene therapy medicinal product (Fig. 14.1). For example, tabelecleucel (Ebvallo®) consists of allogeneic Epstein-Barr virus (EBV) specific T cells and is registered as a cell therapy medicinal product, whereas Zalmoxis® also consists of allogeneic T cells. Still, these were genetically modified with a retroviral vector to express the human low-affinity nerve growth factor receptor and the herpes simplex virus type 1 (HSV-1) thymidine kinase enzyme. This product was therefore registered as an ex vivo gene therapy medicinal product. Similarly, products based on ex vivo genetic modification of autologous T cells to express a chimeric antigen receptor (CAR-T cells) are categorized as ex vivo gene therapy medicinal products.

Classification

Besides the classification of ATMPs based on regulation as described above, ATMPs can be classified in many other ways, e.g., by:

1. The therapeutic indication they aim to address, e.g., neurological, cardiovascular, or ophthalmological.
2. Whether they comprise cells and/or tissues (see Fig. 14.2):
   (a) derived from and administered to the same human individual (autologous = autogenic), hence the donor = the recipient (patient);
   (b) derived from a human (healthy) donor, who is different from the patient (allogeneic);
   (c) derived from an animal (xenogeneic; see Chap. 9), e.g., porcine islets to treat diabetes mellitus (DM).
3. The potency of the cells, i.e., omnipotent, pluripotent, multipotent, oligopotent, and unipotent (see Table 14.3).
4. The in vivo mode of action (i.e., pharmacological, immunological, metabolic, or regenerative; i.e., regenerate, repair, or replace a human tissue).
5. Their underlying technology, as described in this chapter (Mount et al. 2015):
   (a) somatic cell technologies;
   (b) cell immortalization technologies;
   (c) ex vivo gene modification of cells using viral vector technologies;
   (d) in vivo gene modification of cells using viral vector technologies (see this chapter);
   (e) genome editing technologies;
   (f) cell plasticity technologies;
   (g) three-dimensional technologies;
   (h) combinations of the above technologies.
6. The cell types, e.g., MSCs, dendritic cells (DCs), and T cells.

ATMPs: Possible Mode of Action(s)

The in vivo mode of action(s) (MoA(s)) of an advanced therapy depends on the type of cell/tissue, the ex vivo manipulations performed on the cells/tissue in the manufacturing facility, e.g., genetic modification, the route of administration and the in vivo environment of the cells/tissue:

Table 14.2 EU-ATMP classification definitions according to the EU pharmaceutical legislation, adapted from Smith et al. (2015)

ATMP classification	Definition	Examples
Gene therapy medical product (GTMP)	A GTMP is a biological medicinal product (*excluding vaccines)* that: (a) Contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence and; (b) Its therapeutic, prophylactic or diagnostic effect relates *directly* to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence Gene therapy medicinal products shall not include *vaccines against infectious diseases* (see Chap. 14), which have their own set of vaccine specific guidances	Glybera® (see Chap. 16); Kymriah® (autologous CD19$^+$ CAR-T cells); Strimvelis® (genetically modified autologous CD34$^+$ cells)
Somatic cell therapy medicinal product (SCTMP)	A SCTMP is a biological medicinal product which fulfils the following two characteristics: (a) Contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor (b) Is presented as having properties for or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues	Alofisel® (allogeneic MSCs); irradiated plasmacytoid dendritic cell line (allogeneic) loaded with peptides from tumor antigens
Tissue engineered product (TEP)	A TEP is a biological medicinal product that meets the following two characteristics: (a) Contains or consists of engineered cells or tissues, and (b) Is presented as having properties for, or is used in or administered to human beings with a view to regenerating, repairing or replacing a human tissue A TEP may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or non-viable. It may also contain additional substances, such as cellular products, biomolecules, biomaterials, chemical substances, scaffolds or matrices. Products containing or consisting exclusively of non-viable human or animal cells and/or tissues, which do not contain pharmacological, immunological or metabolic action, are excluded from this definition. Cells or tissues shall be considered "engineered" if they fulfill at least one of the following conditions: (a) The cells or tissues have been subject to substantial manipulation, so that biological characteristics, physiological functions or structural properties relevant for the intended regeneration, repair or replacement are achieved (b) The cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor	Spherox® (autologous chondrocytes); Holoclar® (autologous corneal epithelial cells, which contain stem cells)
Combined ATMP	A combined ATMP fulfills the following conditions: (a) It must incorporate, as an integral part of the product, one or more medical devices or one or more active implantable devices, and (b) Its cellular or tissue part must contain viable cells or tissues, (c) Its cellular or tissue part containing non-viable cells or tissues must be liable to act upon the human body with action that can be considered primary to that of the devices referred to	Allogenic adipose derived regenerative cells encapsulated in hyaluronic acid (TEP + device)[a]; encapsulated allogeneic cells secreting GM-CSF + irradiated autologous tumor cells (GTMP + device)

CD19$^+$ (*CAR-T cells*) cluster of differentiation (CD) 19 "chimeric antigen receptor T cells", CAR-T cells, *GM-CSF* granulocyte-macrophage colony-stimulating factor

[a] Hassan et al. (2013)

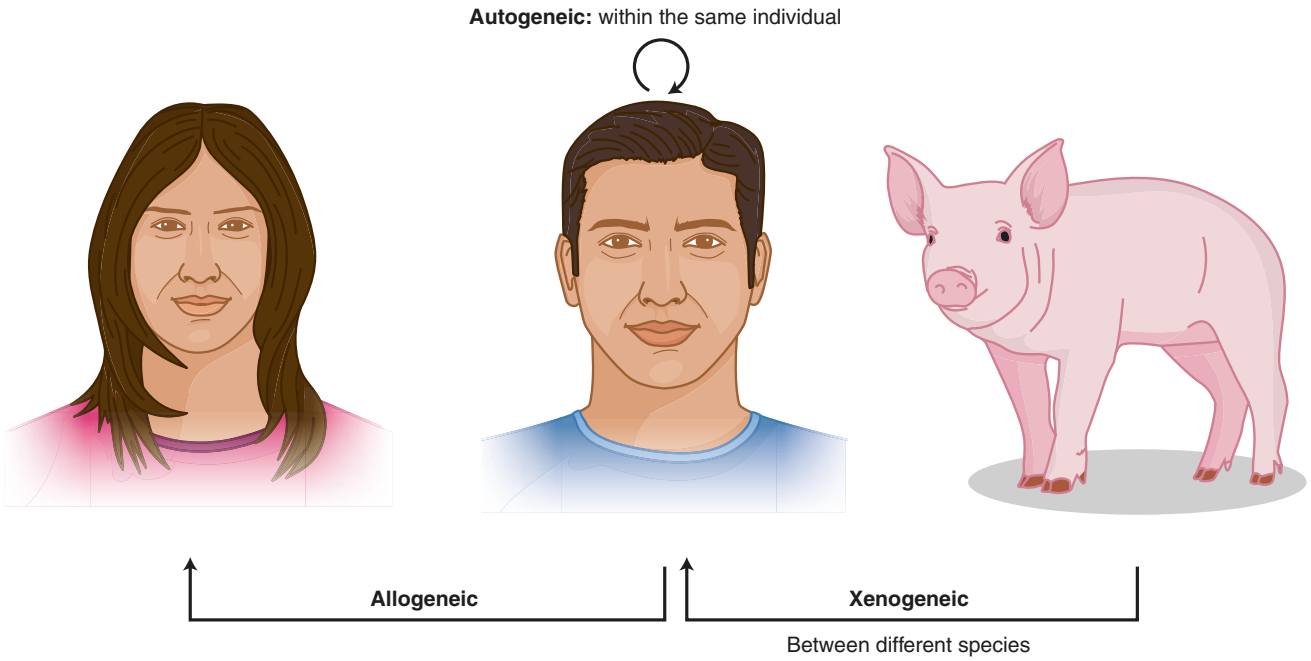

Fig. 14.2 Types of transplants/advanced therapy cell source

Table 14.3 Categorization of stem cells on their potency

Stem cell potency	Explanation and examples
Totipotent (or omnipotent) stem cell	Can differentiate into all embryonic and extraembryonic cell types (i.e., in humans they give rise to the foetus, umbilical cord, and the placenta: morula's cells (0–5 days old embryo)
Pluripotent stem cell	Can differentiate into all three germ cell types (endoderm. mesoderm, or ectoderm lineage) but not the placenta and umbilical cord, and subsequently into all embryonic cell types: ESCs, iPSCs
Multipotent stem cell	Can differentiate into closely related cells, such as all cells in a particular organ: MSCs. other adult (=somatic) stem cells
Oligopotent stem cell	Can differentiate into a restricted closely related group, such as a hematopoietic progenitor cell that can produce a subset of blood cell types, such as B and T cells; vascular stem cell that has the capacity to become both endothelial or smooth muscle cells
Unipotent stem cells (or precursor cell)	Have the property of self-renewal but can only give rise to cells of their own lineage, such as muscle or skin stem cells. This distinguishes these cells from real stem cells as they do not differentiate into other cell phenotypes

1. Pharmacological: cells/tissue release molecules such as cytokines and growth factors upon interaction with their/its environment. An example is the immunoregulatory effect of MSCs. E.g., darvadstrocel (Alofisel®) contains expanded adipose-derived MSCs, which, once activated, impair the proliferation of lymphocytes and reduce the release of pro-inflammatory cytokines at inflammation sites in patients with luminal Crohn's disease. This immunoregulatory activity reduces inflammation and may allow the healing of the tissues around the fistula tract;
2. Regenerative: ex vivo manipulated cells/tissue regenerate, repair, or replace a diseased or damaged human tissue. E.g., to replace damaged ß-cells of a patient suffering from DAM type I, whereby human (h)ESCs are ex vivo differentiated into pre-ß cells, loaded into a device, and administered subcutaneously.
3. Immunological: cells of the immune system are ex vivo activated. E.g., cytotoxic T lymphocytes (CTL) or genetically modified cells (e.g., CAR-T cells) activate the patient's own immune system upon administration, e.g., to treat cancer.

(Dis)similarities with Recombinant Therapeutic Proteins and Other Biologicals

Although ATMPs fall within the group of biologicals, there are substantial differences in the area of chemistry, manufacturing, and controls (CMC), nonclinical, clinical, regulatory, and costs/reimbursement compared to recombinant proteins, vaccines, and plasma-derived medicinal products. This is summarized in Table 14.4 and further discussed in this chapter.

Table 14.4 Example of differences between advanced therapies and biopharmaceuticals

Category	Characteristic	Advanced therapies	Other biopharmaceuticals
Non-clinical	Animal models	Often no relevant animal models to predict safety and particularly efficacy in humans available	Relevant animal models to predict safety/efficacy often available
	Safety testing	Tumorigenicity testing may be needed (stem cell derived products)	N.A.
	ADME/pharmacodynamic studies	Often not possible/relevant	Generally performed
Clinical	Disease pathway(s) and mode of action	Often not well understood	Well understood
	First in human trials	Always in patients	Often in healthy volunteers
	PK/PD studies	Often not feasible/relevant	Performed
	Route of administration	Often IV infusion, sometimes local injection, e.g., into tumor, subretinal space of eye; spinal cord: brain: intra-dermal	IV infection or infusion. SC, intradermal. IM
	Patient monitoring	Often long-term follow-up (10–20 years)	Short term follow-up
	Track and traceability	From donor start material (tissue/cell) through manufacturing process to patient and vice versa	From starting material through manufacturing process to patient
Quality/CMC	Product group	Heterogeneous	Less heterogeneous
	Type of formulation	Often a dispersion/suspension of cells	Often a solution (liquid or reconstituted lyophilizate); sometimes emulsion or suspension (vaccines)
	Dose	Mostly number of (viable) cells/kg body weight or cm^2 tissue	Usually milligram range for proteins; microgram range for vaccines: or defined as units activity/mg
	Manufacturing process	Often continuous process, no designated drug substance	Often discontinuous process, designated drug substance and drug product
		Often open and manual process steps: no platform technologies yet. automation in its infancy	Closed and mostly automated process steps: platform technologies
		Often aseptic manufacture, no sterilization possible (no viral removal and/or inactivation steps) due to viability of cell/tissue	Viral removal and/or inactivation steps; sterilization (mostly through ≤0.2 micron filtration)
	Batch definition	Often one batch for one to few patients; off-the-shelf products less common	Off-the-shelf (one batch for multiple patients)
	Safety	Risk for transmission of human viral infections from donor to patient; animal and human derived raw materials and excipients	Risk extremely low due to viral removal/inactivation steps; chemically defined raw materials and excipients
	Product storage and supply	Sometimes 2–8 °C or room temperature—short shelf-life; often vapor phase of liquid nitrogen (at <−150 °C)—longer shelf-life (months–years)	Mostly 2–8 °C and longer shelf-life (years)

Table 14.4 (continued)

Category	Characteristic	Advanced therapies	Other biopharmaceuticals
Regulatory	Landscape	Evolving regulatory landscape	Established regulatory landscape
	Guidances	Specific "advanced therapy" guidances	Guidances for biologicals and vaccines
	Classification	Product classification and product terminology not harmonized globally	Product classification and product terminology mostly harmonized globally
Ethics	Uncontrolled access to non-approved product	Stem cell tourism	Illicit use of biopharmaceuticals
	Acceptability starting material (tissue/cells)	Use of human embryos to manufacture human embryonic stem cell based product not allowed in some countries	N.A.
Reimbursement	Costs	Very high (20,000–1,000,000 Euros) per treatment	Medium–high (500–5000 Euros) per injection

N.A. not applicable, *ADME* absorption, distribution, metabolism, elimination, *PK* pharmacokinetics, *PD* pharmacodynamics, *IV* intravenous, *SC* subcutaneous, *IM* intramuscular, *CMC* chemical, manufacturing, and controls

Part A: Technologies for Cell Therapy and Tissue Engineering

Although ATMPs can be classified by the regulatory regime to be applied (see above), the diversity of this new group of biologicals may be better illustrated by the underlying technology and their potential as therapeutics (Mount et al. 2015). Below, these technologies are briefly discussed with examples of products in clinical development or approved for commercial use.

Somatic Cell Technologies

This technology involves the use of adult stem cells, also known as somatic stem cells. The fundamental property of a stem cell is the capability to multiply, i.e., it has the self-renewal capacity, which is the ability to go through numerous cycles of cell division while maintaining the undifferentiated state and to give rise to a variety of differentiated cells. Stem cells can be characterized by their potency, which is the ability to differentiate into specific cell types. The more cell types it can generate, the higher the potency (Table 14.3). Different stem cells exist (Table 14.5) and can be isolated from embryos, blood cords, tissues, and organs, or they can be derived from differentiated somatic cells (mostly skin fibroblasts) by inducing pluripotency via forced expression of specific transcription factors, the so-called induced pluripotent stem cells (iPSC; see section "iPS Cell Technology").

Adult stem cells are known to be present in many, if not all, individual organs in adults and are generally thought to be "multipotent"; that means that they have the ability to differentiate into all cell types within one particular lineage, i.e., they can give rise to the cells found in their organ of origin, but not in other organs (Fig. 14.3). In tissues, also in brain tissue in the subventricular zone and in the dentate gyrus (part of the hippocampus), they exist in an organized environment of supporting cells that define the architecture of the "stem cell niche" (Scadden 2006). A hallmark of adult stem cells is their ability to "self-renew" both in vivo and ex vivo and that they undergo asymmetric cell division.

This means that when they divide, they usually give rise to two different cells, one identical stem cell and the other a partly differentiated progenitor cell (Fig. 14.4). The common pattern in adult tissues is that the resulting progenitor cells are capable of expansion by symmetric division and can subsequently differentiate into the various cell types needed for repair or replenishment of the relevant tissue. Adult stem cells include chondrocytes, HSCs, MSCs, skin stem cells, and immune cells (see Table 14.5). Isolation of adult stem cells from organ-tissues is a challenge because only very small numbers of stem cells reside, and once removed from the body, these cells grow to senescence, a state in which cells stop dividing but do not die. Thus, obtaining large quantities of stem cells is difficult. Also, the separation of stem cells from other (unwanted) cell populations is far from trivial. For some products, master and working cell bank (MCB and WCB) strategies are applied. Still, genetic and phenotypic stability, i.e., certain markers present on the cell surface, must be closely monitored.

Adult Stem Cells Used as Transplant Product

Adult stem cells have been used since the 1950s to treat cancers of blood cells as one of the components of bone marrow

Table 14.5 Origin, characteristics, and uses of "stem" cells

Type of stem cell	Origin	Characteristic potential (see also Table 14.6)	Application
Adult (=somatic) stem cells	Exist in small number in many tissues, often in a well-defined and supportive niche	Multipotent: Give rise to cells of the relevant tissue or local environment	Neural stem cells and limbal stem cells in pre-clinical and clinical development
MSCs (a group of adult stem cells)	A collective term for cells tram mesodermal lineage, sourced from stromal or connective tissue (e g., bone marrow, adipose tissue, and umbilical cord tissue)	Multipotent: A heterogeneous pool of cells. They have a "stem cell-like" character and can differentiate into cells of connective tissues, e.g., chondrocytes, osteoblasts, and adipocytes, but they have also been reported to give rise to many other unrelated cell types	Pre-clinical development & clinical PI-III trials; commercial (Prochymal® and Alofisel®)
Cord blood-derived MSCs (primitive stem cells, somewhere between ESCs and mature adult stem cells)	A specific source of MSCs. Extracted at birth from umbilical cord blood	Multipotent: Yet to be fully determined. Potentially they could be a source of many cell types for individual patients	Private cell banks are established for cryopreservation of cord blood samples; pre-clinical development and clinical phase I/II trials
ESC (no adult stem cells)	Result from ex vivo culture of the inner cell mass of a blastocyst (embryoblast = 5–9 days old embryo)	Pluripotent	Vital source of differentiated cells for different research applications and clinical first in human (FIH) trials ongoing
iPSC (no adult stem cells	Derived by reprogramming of somatic cells (often skin fibroblasts) taken from an adult biopsy	Pluripotent, although methods for full reprogramming are still in development	From autologous source for disease modelling, drug screening including toxicity testing, and FIH trial; pre-clinical development and plans for human leukocyte antigens (HLA)-matched allogeneic iPSCs for FIH trial; research is ongoing with allogeneic iPSCs eliminating HLA-class I expression using genome editing technologies to generate universal cell

transplants (Santos 1983). This procedure involves whole-body irradiation to kill malignant cells in multiple myelomas and leukemia. The patient then receives a bone marrow transplant, not in itself a stem cell product, but the transplant contains a few HSCs which subsequently home to the bone marrow stem cell niches and begin to replenish the blood (Fig. 14.5). Rejection and graft-versus-host disease (GvHD) are still threatening complications of this form of therapy.

Adult Stem Cells for Clinical Application: Immune Cells

Immune cell types currently investigated for their therapeutic value, mostly in the field of cancer, are DCs (see also Chap. 15), tumor-infiltrating lymphocytes (TILs), γδ T cells, regulatory T cells (Tregs), macrophages, and viral reconstitution T cells. Both autologous and allogeneic cells are used as cell sources. These immune cells have a highly specific mode of action and are in different stages of clinical development.

Adult Stem Cells for Clinical Application: MSCs

MSCs, sometimes called multipotent stromal cells or mesenchymal stem cells, have generated considerable interest in cell therapy applications (Bianco et al. 2008). However, the description of the cells, their source, and manufacturing processes are quite heterogeneous. MSCs can, e.g., be isolated from bone marrow, adipose tissue, corneal epithelial cells, and from a gelatinous substance within the umbilical cord (Wharton's jelly) and umbilical cord blood. MSCs differentiate into various phenotypes, including chondrocytes, osteoblasts, and adipocytes. Due to their pleiotropic properties, e.g., growth factors and chemokines producing, anti-apoptotic, angiogenetic, anti-fibrotic, and neuroprotective, have been extensively tested in preclinical models. Hundreds of Phase I-III clinical trials have been performed and are ongoing globally in a wide variety of indications: cardiovascular diseases, GvHD, brain and neurological disorders, muscle, bone, and cartilage diseases, lung and bronchial dis-

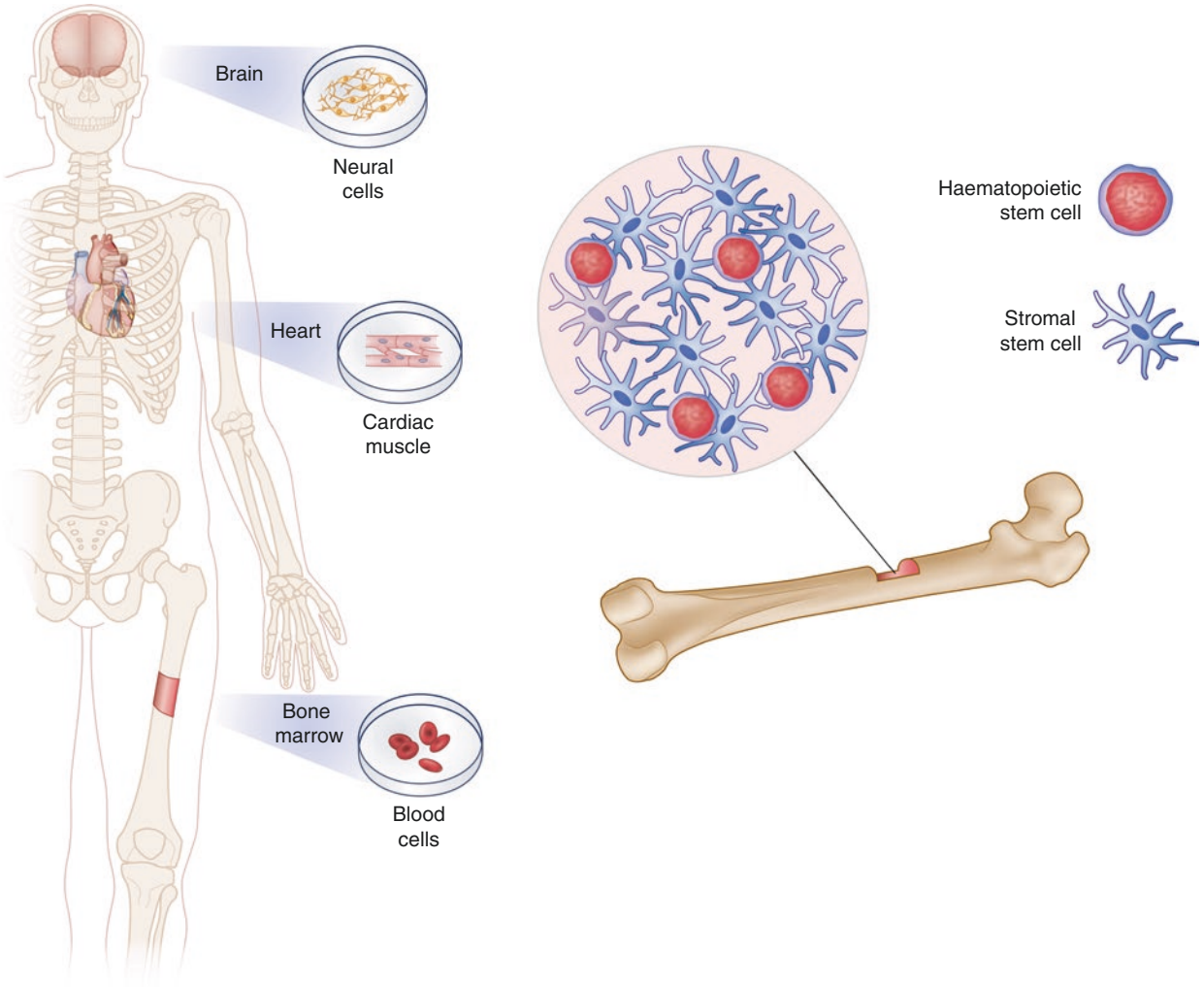

Fig. 14.3 Adult stem cells are present in many tissues in specific stem cell niches, giving rise to a specific group of cells found in the relevant tissue. The examples shown have been studied in detail but adult stem cells, yet to be defined, may be present in many other tissues

eases, wounds and tissue restorations, and immune system diseases (Galderisi et al. 2022), see Fig. 14.6.

MSC can be administered locally, e.g., intralesionally, subcutaneously, or intravascularly. While local administration has been found effective in case of local injury, e.g., to treat bone and joint diseases, heart disease, for the repair of muscle and ligament damage, Crohn's fistulas, and even for the repair of ischemic brain tissue, the systemic infusion is preferable in the case of systemic diseases such as GvHD (Kean et al. 2013). Therefore, both autologous and allogeneic cell sources have been studied. Examples of MSC products that have been approved globally are darvadstrocel (Alofisel®) in the EU for the treatment of Crohn's fistulas, remestemcel-L (Prochymal®) in Canada and New Zealand, Holoclar and Alofisel in the United States and Temcell HS in Japan for the treatment of pediatric acute graft-versus-host disease (GvHD) (see also Table 14.6).

Cell Immortalization Technologies

Another technology makes use of immortalized cell lines as starting material for the manufacture of cell-based products. An example of such a cell line is the neural stem cell line CTX0E03, derived from the human fetal cortical brain and genetically modified with a retroviral vector encoding the

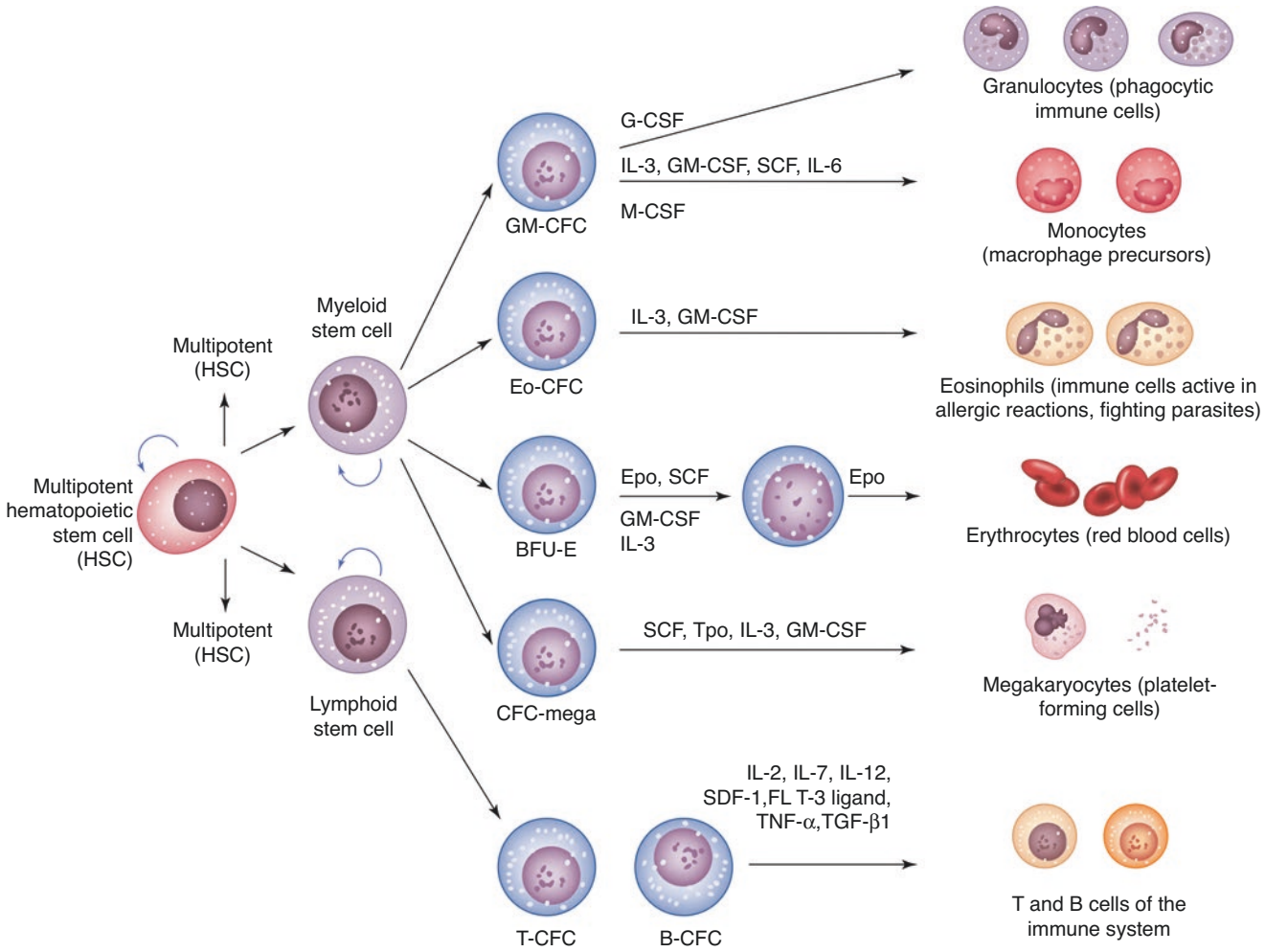

Fig. 14.4 Asymmetric division of adult HSCs, to produce myeloid or lymphoid stem cells, further differentiation to form mitotic progenitors, and subsequently under the control of specific growth factors and cytokines, to form fully differentiated blood cells. The differentiation pathways of the hematopoietic system are better characterized than those of other tissues, but the pattern of differentiation is typical of other tissues. *GM-CSF* = Granulocyte-macrophage colony-stimulating factor, *Eo-CFC* = Eosinophil-leukocyte Colony Forming Cell, *BFU-E* = Bone marrow erythroid progenitor cells, *IL* = interleukin, *SCF* = stem cell factor, *SDF* = stromal cell-derived factor, *TNF* = tumor necrosis factor, *TGF* = transforming growth factor

immortalizing gene, c-mycERTAM (Pollock et al. 2006; Stevanato et al. 2009). Under the conditional regulation by 4-hydroxytamoxifen (4-OHT), this gene enables the large-scale production of the CTX cells using a two-tier cell banking system (MCB and WCB). Clinical testing of the CTX cell-based product is ongoing in a clinical phase II program for stroke. Although cell immortalization technologies have been in development for some time now, this is not a mainstream technology in the pharmaceutical world yet.

Cell Plasticity Technologies

The cell plasticity technology area takes advantage of discoveries that certain cells have the ability to evolve to cell types formerly considered outside their normal differentiation repertoire, i.e., hESCs and iPSCs. This technology has extensive clinical potential due to the high probability of an almost unlimited supply of cells (MCB and WCB approach) and also for the possibility to HLA-match the resulting cell-based product (partly) with the recipient patient. The application of pluripotent stem cells, such as ESCs and iPSCs, goes beyond the administration of cell-based medicinal products and is investigated as a source for tissue engineering and organogenesis (see section "Three-Dimensional Technologies"). In addition, autologous and allogeneic iPSCs are currently extensively used for disease modeling: i.e., patient-specific iPSC-derived cardiomyocytes, cultured in vitro, can be used to identify the genetic basis of cardiac disease, leading to the identification of pharmacogenetic bio-

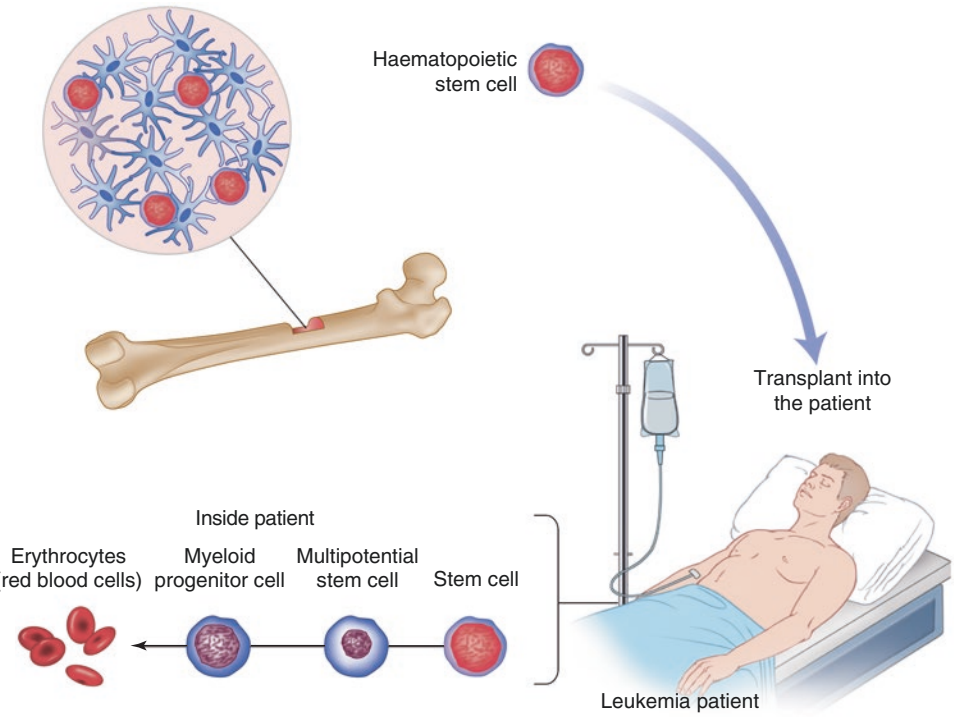

Fig. 14.5 Schematic representation of bone marrow transplantation, a form of stem cell therapy that was first used over 50 years ago. The transplant contains hematopoietic stem cells from the donor. These cells repopulate niches in the recipient bone marrow

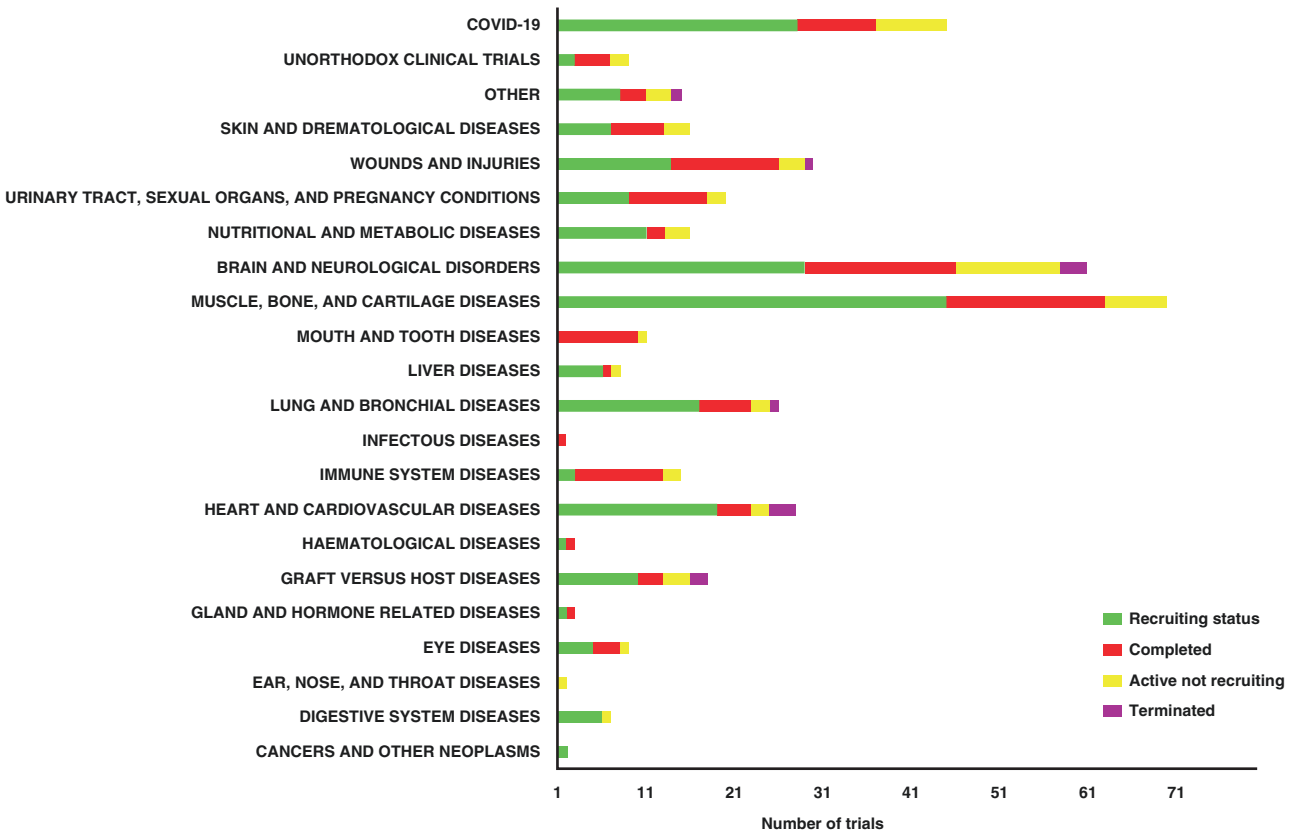

Fig. 14.6 Clinical trials with MSC-derived products and their indication, adapted from Squillaro et al. (2016) (Stem Cell Rev Rep 2022;18: 23–36)

Table 14.6 Available gene and cell therapy medicinal products approved by the EMA and FDA (with the exception of blood cord-derived products; status December 20, 2022)

Brand name	Classification	INN/description	Indication	Company	EMA/FDA approved	Month/year approved
Imlygic™	In vivo GTMP	Talimogene laherparepvec/HSV-1 oncolytic virus containing the cDNA of human GM-CSF	Advanced metastatic melanoma	Amgen	Yes/Yes	December 2015/October 2015
Luxturna™	In vivo GTMP	Voretigene neparvovec/AAV2 vector containing human RPE65 cDNA	Leber congenital amaurosis	Spark Therapeutics	Yes/Yes	November 2018/ December 2017
Zolgensma™	In vivo GTMP	Onasemnogene abeparvovec/AAV9 vector containing the SMN1 transgene	Spinal muscular dystrophy (SMA)	AveXis/Novartis	Yes/Yes	May 2020/May 2019
Upstaza™	In vivo GTMP	Eladocagene exuparvovec/AAV2 vector containing the cDNA of human dopa decarboxylase (DDC)	Patients > 18 months with aromatic L-amino acid decarboxylase deficiencies	PTC Tx	Yes/No	July 2022
Roctavian™	In vivo GTMP	Valoctocogene roxaparvovec/AAV5 vector containing the cDNA of the B-domain deleted SQ form of human coagulation factor VIII (hFVIII-SQ)	Adult patients with severe hemophilia A	BioMarin	Yes/No	August 2022
Hemgenix™	In vivo GTMP	Etranacogene dezaparvovec/AAV5 vector containing the cDNA of human coagulation factor IX	Adult patients with hemophilia B	CSL Behring/ UniQure	No/Yes	November 2022
Adstiladrin™	In vivo GTMP	Nadofaragene firadenovec-vncg/Nonreplicating AV containing the cDNA of human interferon alfa-2b (rAd-IFNa/Syn3)	BCG-unresponsive high-risk nonmuscle-invasive bladder cancer	Ferring Pharmaceuticals A/S	No/Yes	December 2022
Strimvelis™	Ex vivo GTMP	Autologous CD34+ cells transduced with a retroviral vector containing the *ADA* cDNA encoding human adenosine deaminase (ADA)	Severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID)	Orchard Tx	Yes/No	May 2016
Zalmoxis™	Ex vivo GTMP	Allogeneic T cells genetically modified with a retroviral vector encoding for a truncated form of the human low-affinity nerve growth factor receptor (ΔLNGFR) and the herpes simplex I virus thymidine kinase (HSV-TK Mut2)	Add-on treatment for patients who have received a haploidentical hematopoietic stem cell transplant	MolMed S.p.A.	Withdrawn/ No	August 2016
Kymriah™	Ex vivo GTMP	Tisagenlecleucel/autologous CAR-T cells directed to CD19	Acute Lymphoblastic Leukemia (ALL)	Novartis	Yes/Yes	August 2018/ August 2017
Yescarta™	Ex vivo GTMP	Axicabtagen ciloleucel/autologous CAR-T cells directed to CD19	Non-Hodgkin lymphoma: – Large B cell lymphoma – Follicular lymphoma	Kite Pharma	Yes/Yes	August 2018/ October 2017
Zynteglo™	Ex vivo GTMP	Betibeglogene autotemcel/Lentiviral vector containing the cDNA of the human *HBB* gene	Transfusion-dependent beta thalassemia	Bluebird Bio	Yes/Yes	May 2019/ August 2022
Libmeldy™	Ex vivo GTMP	Atidarsagene autotemcel/Lentiviral vector containing the human *ARSA* cDNA encoding arylsulfatase A	Metachromatic Leukodystrophy (MLD)	Orchard Therapeutics	Yes/No	December 2020/
Tecartus™	Ex vivo GTMP	Brexucabtagene autoleucel/autologous CAR-T cells directed to CD19	Mantle Cell Lymphoma Acute Lymphoblastic Leukemia (ALL)	Kite Pharma	Yes/Yes	December 2020/July 2020
Breyanzi™	Ex vivo GTMP	Lisocabtagen maraleucel/autologous CAR-T cells directed to CD19	Relapsed or Refractory Large B-cell lymphoma Non-Hodgkin lymphoma Follicular lymphoma	Bristol Meyers Squibb	Yes/Yes	April 2022/ February 2021
Abecma™	Ex vivo GTMP	Idecabtagene vicleucel/autologous CAR-T cells yu76h	Relapsed or Refractory multiple myeloma	Celgene	Yes/Yes	August 2021/ March 2021

Carvykti™	Ex vivo GTMP	Ciltacabtagene autoleucel/autologous CAR-T cells directed to B cell maturation antigen (BCMA)	Relapsed or Refractory multiple myeloma	Janssen Biotech	Yes/Yes	May 2022/ February 2022
Skysona™	Ex vivo GTMP	Elivaldogene autotemcel/autologous CD34+ cells tranduced with retroviral vector containing The cDNA of the human *ABCD1*	Early, active cerebral adrenoleukodystrophy	Bluebird Bio	Withdrawn/ Yes	September 2022
Provenge™	SCTMP	Sipuleucel-T/autologous dendritic cells	Metastatic castrate-resistant hormone-refractory prostate cancer	Dendreon Pharma	No/Yes	Withdrawn/ April 2010
Holoclar™	SCTMP	Ex vivo expanded autologous human corneal epithelial cells containing stem cells	Patients with corneal damage due to (chemical) burns	Holostem Advanced Therapies	Yes/No	February 2015
Spherox™	SCTMP	Spheroids of human autologous matrix-associated chondrocytes	Symptomatic articular cartilage defects of the femoral condyle and the patella of the knee	CO.DON Ag	Yes/No	July 2017
Alofisel™	SCTMP	Darvadstrocel /Mesenchymal stem cells from fat tissue of adult donors	Complex perianal fistulas in patients with Crohn's disease	Takeda Pharma	Yes/No	March 2018
Ebvallo™	SCTMP	Tabelecleucel/allogeneic, EBV-specific T cells from a matched donor	Second-line treatment for transplant recipients who develop, as a result of immunosuppression treatment, Epstein-Barr virus (EBV) associated posttransplant lymphoproliferative disease (PTLD)	Atara Biotherapeutics	Yes/No	October 2022
GINTUIT™	TEP	Allogeneic cultured keratinocytes and dermal fibroblasts in murine collagen	Topical (nonsubmerged) application to a surgically created vascular wound bed in the treatment of mucogingival conditions in adults (venous leg ulcers)	Organogenesis Inc.	No/Yes	March 2012
MACI™	TEP	Matrix-applied characterized autologous cultured chondrocytes	The repair of single or multiple symptomatic, full-thickness cartilage defects of the knee with or without bone involvement in adults	Vericel	Withdrawn/ Yes	December 2016
StrataGraft™	TEP	Allogeneic cultured keratinocytes and dermal fibroblasts in murine collagen	Adults with thermal burns containing intact dermal elements for which surgical intervention is clinically indicated	Mallinckrodt plc	No/Yes	June 2021
Rethymic™	TEP	Human allogeneic processed thymus tissue-agdc	Pediatric patients with Congenital Athymia	Enzyvant	No/Yes	October 2021

markers that support effective and personalized drug therapy and drug discovery including toxicity screening (Sayed et al. 2016).

Embryonic Stem Cells

During the earliest stages of mammalian development, soon after egg and sperm combine, the resulting diploid cells are said to be "totipotent," i.e., they can give rise to both the embryo and placental tissue. At the blastocyst stage of embryogenesis (day 5 in humans), the "inner cell mass" or "embryoblast" is compacted and separated from the surrounding "trophoblast." The latter combines with the maternal endometrium to form the placenta. The inner cell mass can be extracted and grown ex vivo as ESCs, which can give rise to all three germ cell types (mesoderm, endoderm, and ectoderm) and, therefore, potentially any cell type found in the adult (Fig. 14.7).

Mouse ESCs were first isolated in 1981 (Evans and Kaufman 1981; Martin 1981), but it took until 1998 for a similar procedure to be described allowing human ESCs to be grown in culture (Thomson et al. 1998). ESCs can now be grown for many cell divisions, limited only by genetic damage that occurs by mutation after extensive culturing. The pluripotency of ESCs has been demonstrated in mice by injecting cells into a fertilized egg, resulting in the production of chimeric mice, i.e., mice made up of cells derived from both the donor and the injected ESCs, with this technology, transgenic mice for research purposes have been generated. HESCs are currently investigated by a set of cell surface markers (CD markers) and their capacity to differentiate. The criteria for this assessment include the expression of surface markers and transcription factors associated with an undifferentiated state. In addition, extended proliferative capacity, pluripotency, and a euploid karyotype are important characteristics of these cells. Recent advances in human pluripotent stem cell research revealed different subpopulations within stem cell cultures covering a wide spectrum of pluripotent states that hold distinct molecular and functional properties (Goodwin et al. 2020). Moreover, evidence suggests that the epigenetic status of the cells is also a relevant criterion for hESCs. Epigenetic alterations may accumulate when hESCs are cultured in vitro. Therefore, genetic stability over extended periods should be considered as a critical parameter, demonstrating that hESC characteristics do not change over time in terms of karyotype, expression of markers, expression of telomerase and their ability to differentiate into the three germlines (ecto-, meso- and endoderm) (Bar and Benvenisty 2019).

Maintenance and Differentiation of ESCs in Culture

HESCs are grown in the presence of high concentrations of basic fibroblast growth factor-2 (FGF2) and are unresponsive to leukemia inhibitory factor (LIF) (Levenstein et al. 2006). The technical challenge, now that hESCs can be maintained and expanded, is to develop robust methods to control and direct ESC differentiation, so that human cells of any desired phenotype can be obtained (Keller 2005; Murry and Keller 2008) with sufficient purity in terms of the absence of unde-

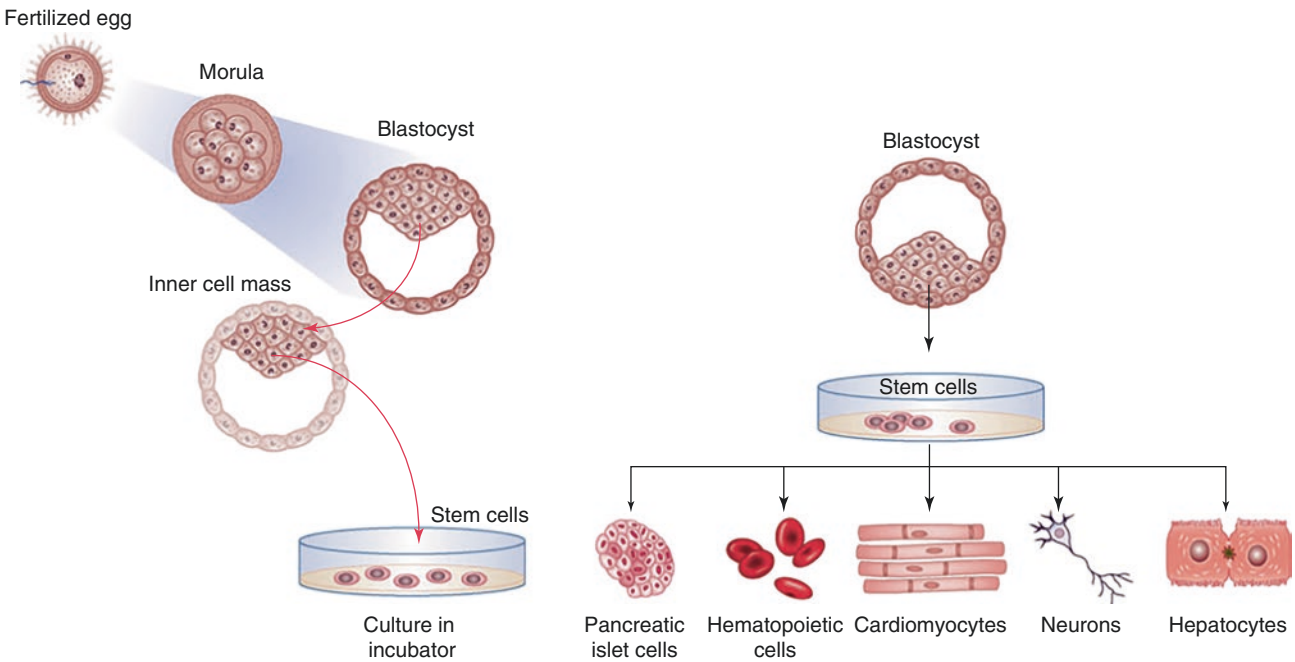

Fig. 14.7 Extraction of the inner cell mass of the blastocyst gives rise to ESCs, which have the capacity to differentiate into all 200+ somatic cell types found in the adult human

sired cells, such as undifferentiated cells, or cells that are capable of de-differentiation into undifferentiated cells or into cells of a different lineage, either of which could cause tumor formation after implantation, both at the site of administration or elsewhere in the body after cell migration. This technology has not fully matured yet. Thus far, attention has focused on the differentiation of human ESCs toward products that could be of obvious use for clinical administration, e.g., midbrain dopaminergic neurons for Parkinson's disease, cardiomyocytes for reinforcement of damaged heart tissue, and pancreatic pre-β-islet cells for implantation in Type I DM.

Since they were isolated for the first time, several first-in-human clinical studies with hESC have been initiated for various indications, including neural diseases (Parkinson's disease, spinal injury), heart disease, cancer, and eye diseases (Table 14.7). In an open-label phase I/II study, hESC-derived retinal pigment epithelial cells (RPE) were given subretinally to patients with Stargardt's disease or patients suffering from macular degeneration. Injected RPE cells showed no sign of hyperproliferation or tumor formation, albeit local immunosuppression was needed to prevent rejection. After 22 months, significant improvement in eyesight was reported for 19 out of 27 patients (Schwartz et al. 2015). Whereas researchers clearly have demonstrated their therapeutic potential, the use of hESC remains controversial. The opinions of scientists, regulators, and public are widely divided, from being very supportive to seeking a regulatory ban on hESC research for ethical/religious reasons. Besides these ethical barriers, some scientific barriers still need to be overcome. Progression towards clinical applications is hampered due to safety concerns, specifically immunogenicity and the unknown potential of undifferentiated escapees for teratomas.

ESC Somatic Cell Nuclear Transfer (Therapeutic Cloning)

An alternative, particularly when an HLA-donor match cannot be found, is to produce ESCs for individual patients, by somatic cell nuclear transfer (SCNT) (Wilmut et al. 2002). This process, also known as "therapeutic cloning," involves the implantation of a cell nucleus from the patient (i.e., genomic DNA extracted from a skin biopsy) into a human egg, which has undergone the removal of its own DNA. The environment in the enucleated egg can reprogram the DNA from the patient, removing epigenetic marks and restoring the DNA to an embryonic state. In addition, the development of an inner cell mass in the egg, after a period of incubation, allows extraction of ESCs that have the patient's exact genotype. These cells could be used subsequently for the production of implants for cell therapy (Fig. 14.8).

Somatic cell nuclear transfer (SCNT), moving nuclear DNA from a donor cell to an enucleated recipient cell to create an exact genetic match of the donor, is an inefficient process. Most eggs that have undergone SCNT cannot completely reprogram the donor DNA, and the surrogate pregnancy is usually unproductive. Moreover, even when the pregnancy comes to term, the cloned offspring are known to carry many epigenetic marks that may compromise normal development.

Given that defects are known to occur after SCNT, the subsequent derivation of cells for clinical uses might also be prone to failure due to defects in ESC differentiation. There is insufficient data available at this stage to judge whether this will be a limitation in practice. However, significant ethical concerns have limited the practice of SCNT. A human egg donor is required, and unless the process becomes more efficient, women who are prepared to donate eggs would need to provide several eggs to produce a single ESC line. There is concern that women could be exploited, particularly women from low economic backgrounds, and as a result, SCNT is not supported by government funding at present in most countries. A restricted number of ESC lines have been produced using spare eggs from in vitro fertilization programs, but SCNT remains a controversial topic and is subject to legal constraints that vary from country to country for mainly biomedical ethical reasons. An alternative source of cells for clinical application is umbilical cord blood stem cells, which are now being banked at childbirth (i.e., in a biobank), at least in private practice, and the first clinical trials have been initiated. Whether cord blood cells can be harnessed to produce all cell phenotypes is not clear at present (see also above "cord blood-derived MSCs"). However, many of the ethical issues surrounding SCNT and uncertainty of cord blood stem cell potency (in vivo activity), may become irrelevant if the promise of iPSCs can be fulfilled.

IPS Cell Technology

Initially, work on pluripotent stem cells (PSCs) was conducted using hESCs; however, the requirement to destroy early-stage embryos in the process of ESC derivation makes their use ethically controversial. In addition, practical considerations hinder their medical applications because any cells or tissues generated from hESCs, by definition, would be allotransplants into the recipient patient (see above).

However, since the discovery of Takahashi and Yamanaka in 2006 that differentiated somatic cells (in particular fibroblasts) can be reprogrammed to produce pluripotent cells by inducing the expression of four transcription factors (Sox2, Oct4, Klf4, and cMyc), the need for human embryo's as a source of pluripotent stem cells has become obsolete (Takahashi and Yamanaka 2006; Takahashi et al. 2007). Over the last decades, induced pluripotent stem cells (iPSC) have exploded, and the technology is now in use in hundreds of

Table 14.7 List of reported and registered hESC-based clinical trials. Adapted from Golchin et al. (2021)

	Indication	Phase	Subject	Start/finish date	Country	Reference
Neural diseases	Spinal cord injury	Phase 1	5	2010–2013	USA	Scott and Magnus (2014)
	Amyotrophic Lateral Sclerosis (ALS)	Phase 1 and Phase 2	21	2018–2020	Israel	NCT03482050
	Parkinson diseases	Phase 1 and Phase 2	50	2017–2020	China	NCT03119636
Heart diseases	Severe heart failure	Phase 1	10	2013–2018	France	NCT02057900
Diabetes	Diabetes type 1	Phase 1 and Phase 2	69	2014–2021	USA	NCT02239354
Reproductive insufficiency	Primary ovarian insufficiency	Phase 1	28	2019–2021	China	NCT03877471
	Infertility	Not applicable	240	2017–2020	China	NCT02713854
	Infertility	–	40	2002–2025	Israel	NCT00353197
Eye diseases	Retinitis pigmentosa	Phase 1	10	2019–2020	China	NCT03944239
	Retinitis Pigmentosa	Phase 1 and Phase 2	12	2019–2021	France	NCT03963154
	Macular degenerative disease	Phase 1 and Phase 2	36	2018–2029	UK	NCT03167203
	Dry Age Related Macular Degeneration Disease (Dry AMD)	Phase 1 and Phase 2	10	2017–2020	China	NCT03046407
	Dry AMD	Phase 1 and Phase 2	16	2015–2023	USA	NCT02590692
	AMD	Phase 1 and Phase 2	10	2018–2020	China	NCT02755428
	Stargardt's Macular Dystrophy (SMD)	–	12	2013–2019	UK	NCT02941991
	Dry AMD	Phase 1	3	2016–2019	South Korea	NCT03305029
	AMD	Phase 1 and Phase 2	12	2012–2020	South Korea	NCT01674829
	Outer retinal degenerations	Phase 1 and Phase 2	18	2015–2019	Brazil	NCT02903576
	SMD	Phase 1 and Phase 2	12	2011–2015	UK	NCT01469832
	SMD	Phase 1 and Phase 2	13	2011–2015	USA	NCT01345006
	Advanced Dry AMD	Phase 1 and Phase 2	13	2011–2015	USA	NCT01344993
	Age-related macular degeneration	Phase 1 and Phase 2	24	2015–2024	Israel	NCT02286089
	SMD	Phase 1	3	2012–2015	South Korea	NCT01625559
	SMD patients	–	13	2012–2019	USA	NCT02445612
	AMD	–	11	2012–2019	USA	NCT02463344
	AMD	Phase 1	2	2015–2019	UK	NCT01691261
	Retinal pigment	–	2	2016–2020	UK	NCT03102138
	Macular degeneration diseases	Phase 1 and Phase 2	15	2015–2019	China	NCT02749734
Immunotherapy	Non-small cell lung cancer	Phase 1	48	2018–2022	UK	NCT03371485
Injury	Meniscus injury	Phase 1	18	2019–2020	China	NCT03839238

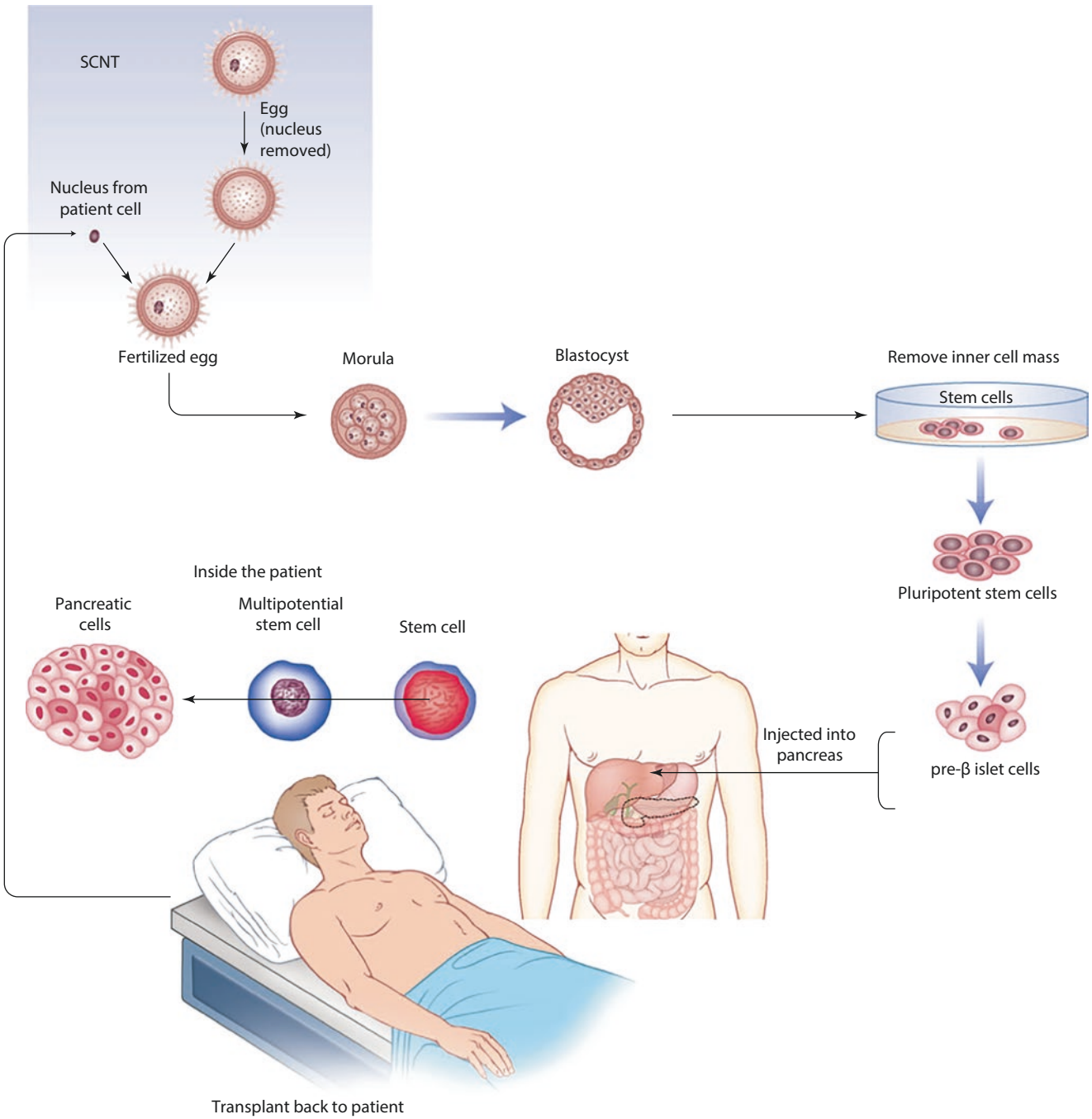

Fig. 14.8 Schematic diagram of the production and clinical use of cell therapies derived using somatic cell nuclear transfer (therapeutic cloning). The example given is for possible treatment of Type I insulin-dependent DM. The final maturation of the pre-β islet cells occurs in the patient's body

stem cell biology laboratories around the world and has been tested for therapeutic applications in clinical trials (Figs. 14.9 and 14.10). The four genes initially identified can be partly substituted by alternatives, and several experiments have shown that integrated lentiviral constructs can be avoided to reduce safety concerns, by using nonviral plasmids (Jia et al. 2010), miRNA and mRNA (Yang et al. 2011; Liu and Verma 2015), protein transduction, and even by substituting some of the factors with small molecules (Yuan et al. 2011).

Considerable effort has been directed at investigating how iPSCs differ from ESCs and whether reprogramming is complete enough to produce truly pluripotent cells. True pluripotency is difficult to demonstrate unequivocally in human iPSCs, so the development of methods to measure the extent

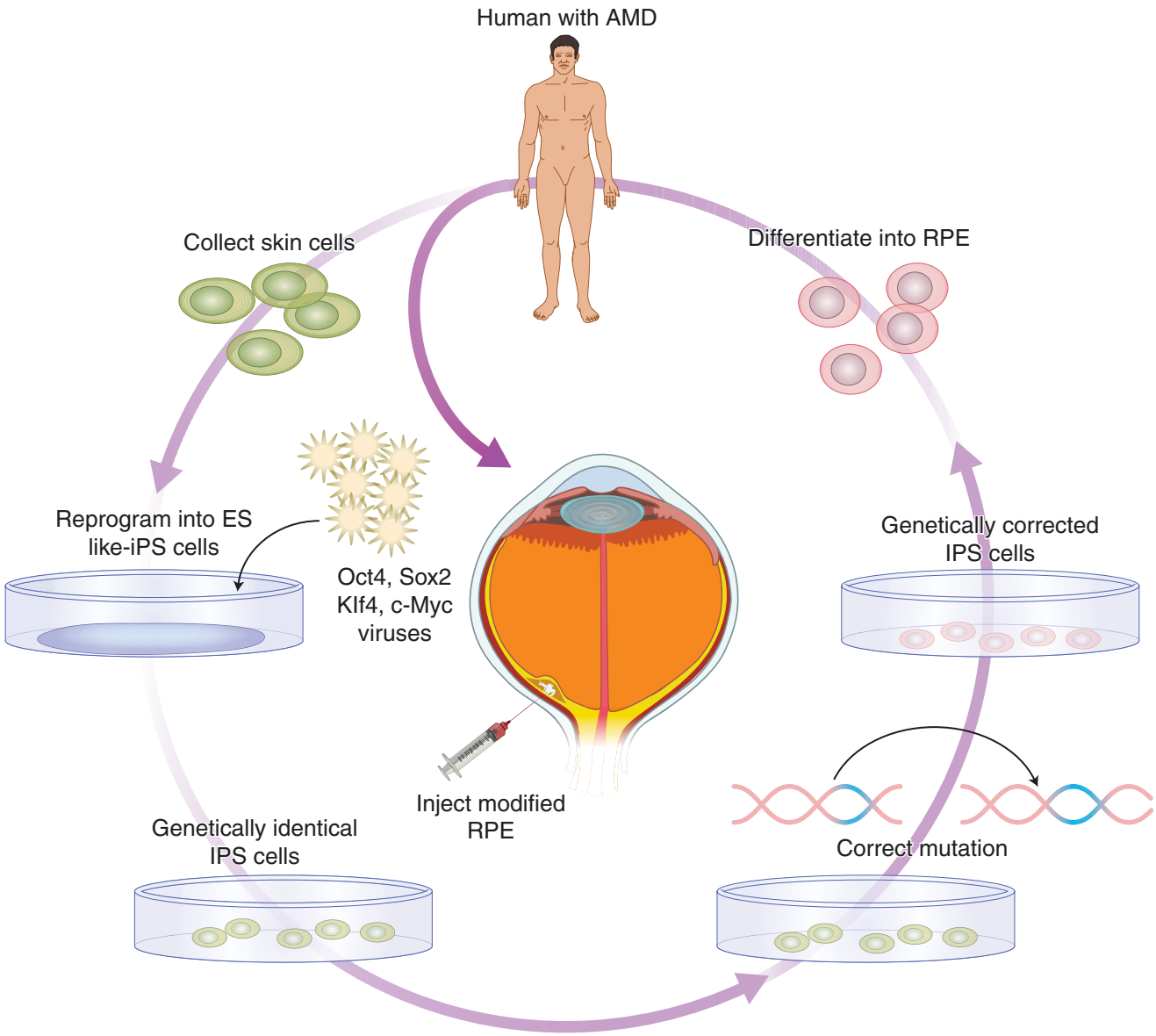

Fig. 14.9 Method used to produce iPS cells, correct a genetic defect responsible for AMD, and implant the corrected stem cells into humans to cure AMD

of reprogramming will be important for practical applications. There are indications that iPSCs can have chromosomal defects and are not fully reprogrammed (Chin et al. 2010). Female human iPSCs appear to maintain the inactivated X chromosome that was present in the skin fibroblasts, although this has not been a problem with mouse iPSCs (Tchieu et al. 2010).

In recent years, progress has been made with improved culture techniques and differentiation protocols, which resulted in safer and clinically relevant iPS cells with lower tumorigenic risk. Various clinical trials with iPSC are being conducted worldwide with Japan and the US being the front-runners (Fig. 14.10) (Kim et al. 2022). Similar to hESC, most clinical trials focus on retinal degenerative diseases, with Dr. Takahashi at the Riken Center for Developmental Biology in Japan being a pioneer. She led the first team to successfully transplant autologous iPSC-derived RPE cell sheets into a patient with age-related macular degeneration (AMD) in 2014 (Sayed et al. 2016) and is presently conducting studies on the transplantation of iPSC-derived corneal cells. Despite these individual successes, clinical development of iPSC therapies has been slow, possibly related to potential safety issues associated with these cells, including genomic instability and tumorigenicity (Nori et al. 2015). Further clinical research investigating the long-term safety of using iPSC in regenerative medicine is needed.

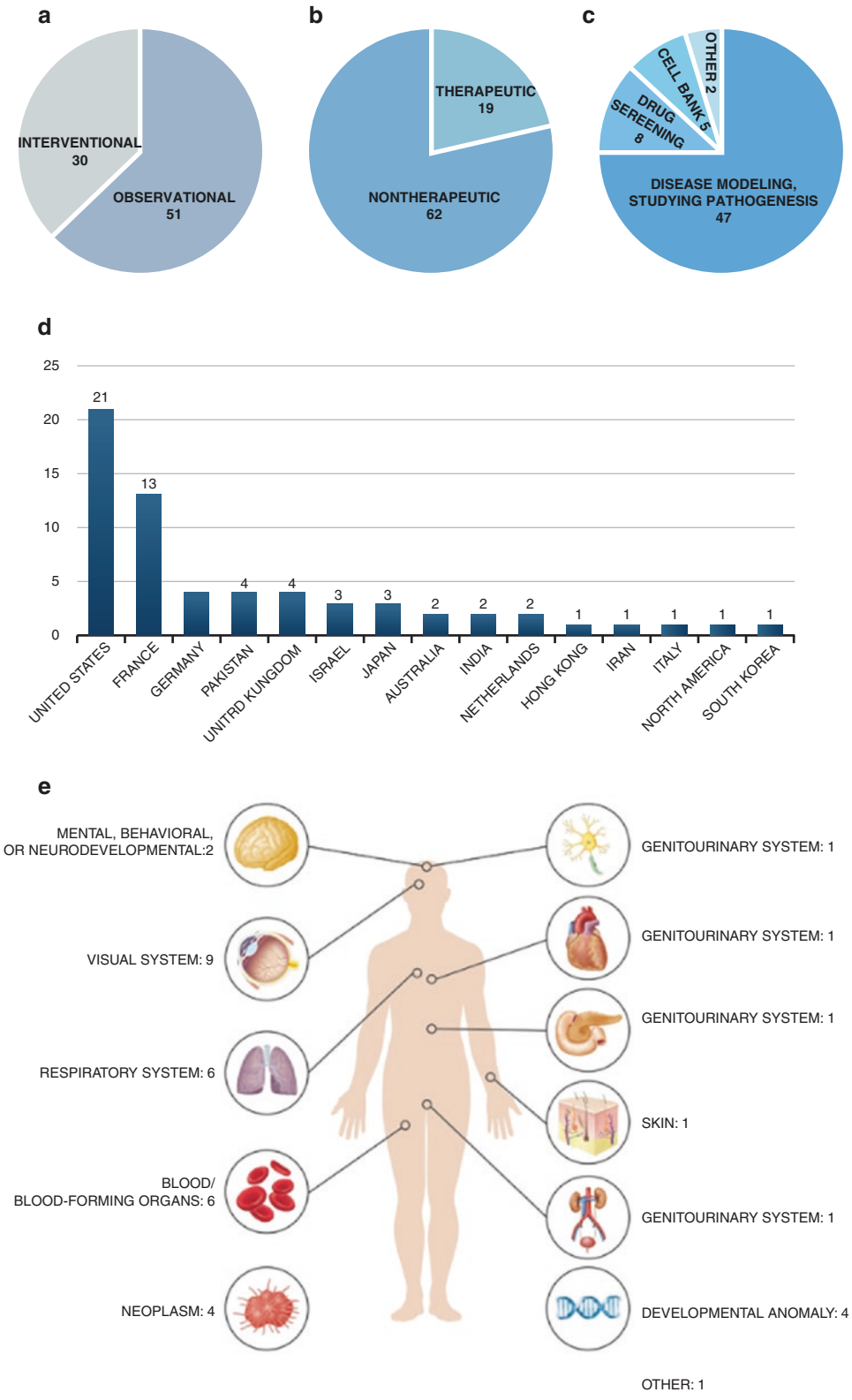

Fig. 14.10 Distribution of clinical trials involving iPSCs accordingly to different categories. (**a**) Initial classification of 81 clinical trials into observational and interventional studies as addressed by the authors. (**b**) New classification of 81 clinical trials into nontherapeutic and therapeutic studies as defined earlier. (**c**) Classification of nontherapeutic clinical trials according to their use. (**d**) Worldwide distribution of nontherapeutic studies. (**e**) Classification of studies according to the category of targeted disease. Adapted from Kim et al. (2022)

Transdifferentiation

Transdifferentiation is the process of converting cells from one lineage into another without going through a pluripotent cell stage, as is the case for reprogramming. Forced expression of genes has been used to convert fibroblasts directly into unrelated differentiated cells by skipping the iPSC stage, but also small chemical compounds can be used for this, although less efficient (Hybiak et al. 2020). Upregulation of apoptosis and cancer-related genes occurs in addition to chromatin remodeling during transdifferentiation. An important criterium of successful transdifferentiation is full morphological and molecular differentiation of both initial and final cells. Different cell types have been generated from fibroblasts, including neurons, hepatocytes, endothelial cells, and cardiomyocytes. The technique used is analogous to that used to derive iPSCs, except that genes associated with the desired somatic cell are expressed instead of pluripotency genes. The realization that cellular phenotypes can be transformed in this way has been met with astonishment and is certainly breakthrough technology. It opens the possibility of performing interconversion in vivo, although it does not allow for the expansion of cells in preparation for an implant. However, direct reprogramming of fibroblasts to neural stem cells, as reported in 2012 (Han et al. 2012; Thier et al. 2012), may be a shortcut to growing neurons. This approach may offer some advantages over the production of neurons by way of iPSCs.

Three-Dimensional Technologies

Another technology, tissue engineering, is combining somatic cell technologies or cell therapy technologies described above, with various types of biocompatible materials to solve structural challenges that are often surgical or immunological in nature. Three-dimensional (3D) technologies, including biomaterial scaffolds, can have many purposes, such as supporting cell viability, induction of cell differentiation, provision of a substrate for cell growth and support of tissue regeneration, provision of the shape, scale, and volume of a desired tissue, provision of growth factors, and encapsulation of cell-based products to protect the product from the host immune system to avoid rejection. This is schematically presented in Figs. 14.11 and 14.12 (Smith and Grande 2015). 3D technologies can be divided into four subtypes of technologies, as shown in Table 14.8. For further reading, see Pina et al. (2019), and Bajaj et al. (2014).

Combinations of the Above Technologies

A combination of the above technologies is currently in preclinical development in the cell therapy area, e.g., the self-

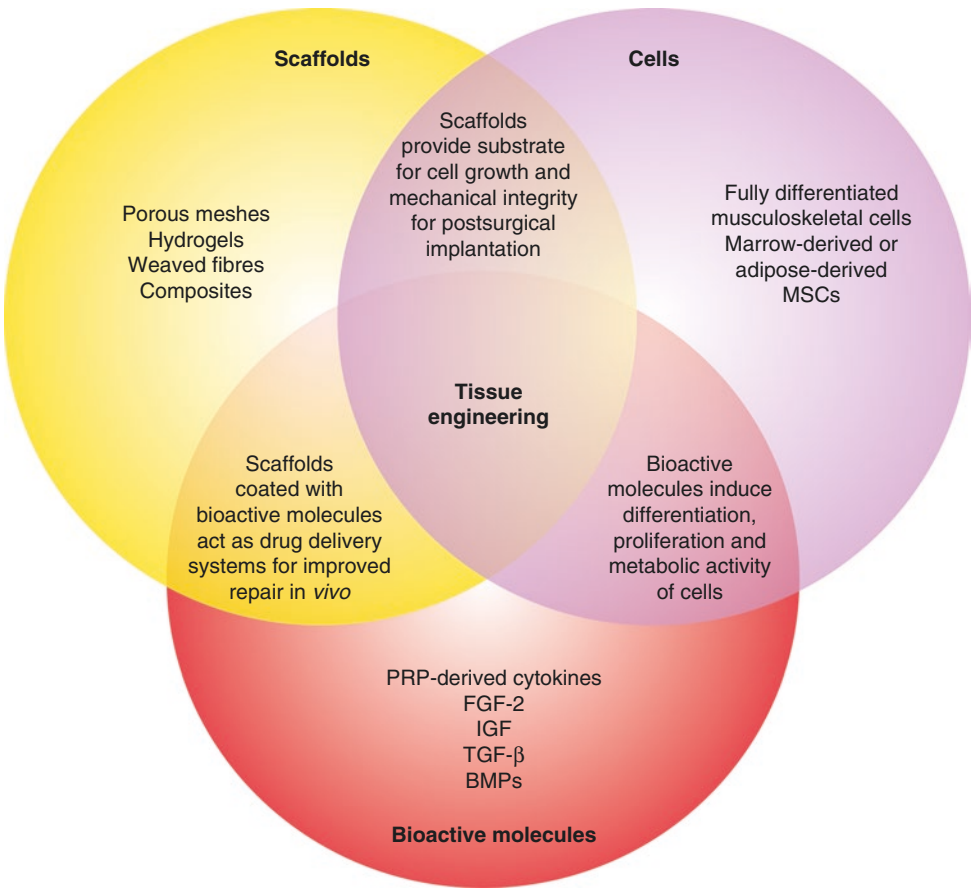

Fig. 14.11 The role of scaffolds in tissue engineering strategies. Scaffolds are an important component of the tissue engineering triad. *BMP* = bone morphogenetic protein, *FGF-2* = fibroblast growth factor 2, *IGF* = insulin-like growth factor, *MSC* = mesenchymal stromal cells, *PRP* = platelet-rich plasma, *TGF-β* = transforming growth factor β. Adapted from Smith and Grande (2015)

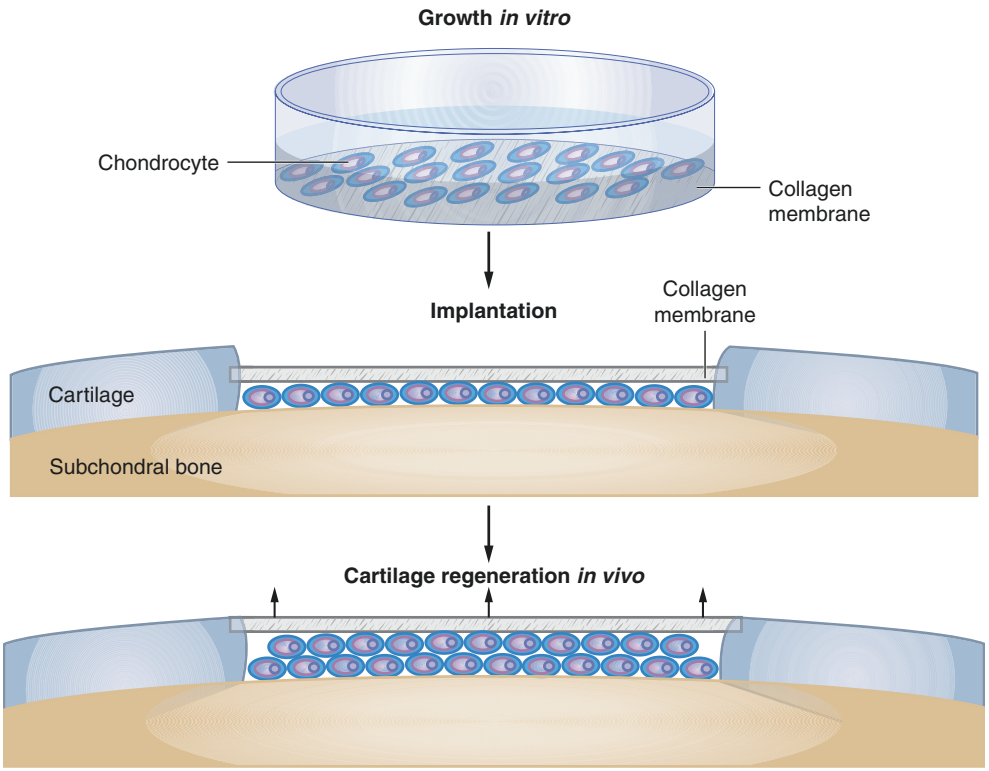

Fig. 14.12 Matrix-assisted chondrocyte implantation (MACI) in cartilage repair. MACI uses chondrocytes that have been seeded into a collagen scaffold and cultured for a period of time prior to surgical implantation, adapted from Smith and Grande (2015)

Table 14.8 3D technologies and examples

Subtype of 3D technologies	Examples of products/organs in pre-clinical or clinical development or commercially used
Simple biomaterials e.g., hyaluronic acid, bone substitutes, alginate-encapsulated islets	Allogenic adipose derived regenerative cells (Keratinocytes) encapsulated in hyaluronic acid to regenerate extracellular matrix-like material to treat corneal blindness; transplantation of pancreatic islets in immune protective alginate capsules to treat DM Type I; MACI® for repair of cartilage defects of the knee (see Fig. 14.13)
3D/shaped scaffolds that provide organ shape and bio-resorbable substrate for cell growth	Bladder; trachea; 3D-printing technologies
Tissue-derived (decellularized) scaffolds that are 3D but with added benefits of native biomechanical strengths and matrix factors	Esophagus; trachea
Smart (second generation) biomaterials that may have thixotropic, thermo-responsive, growth-factor-encapsulating or in situ self-assembly properties	Chitosan and hyaluronic acid are typically used as excipients for thermoset injectable hydrogels encapsulating cells

formation of complex organ buds into organ-like structures, i.e., organoids (Takebe et al. 2015; Brassard and Lutolf 2019).

Part B: Technologies for Gene Therapy

Introduction

Gene therapy aims to treat or cure a disease by inserting, altering, or removing genetic material in affected cells in the human body. The concept of gene therapy arose during the 1960s and early 1970s and was a direct consequence of a series of discoveries made in the preceding years. It started with developing tools to select cells based on a functional trait that could be acquired via the transfer of exogenous DNA. For example, in the mid-1960s, it was demonstrated that cells from patients with Lesch-Nyhan syndrome, which lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT), could be rescued upon uptake of exogenous DNA encoding HPRT, when grown in hypoxanthine-aminopterin-thymidine (HAT) medium which is selective for

cells expression HPRT. Despite this proof-of-concept that exogenous DNA can be expressed in mammalian cells, the genetic transformation was inefficient. Methods to facilitate the delivery of exogenous DNA were developed, including the calcium phosphate chemical transfection method. Later, work on polyomaviruses, papovaviruses, and retroviruses provided the tools for more efficient transformation. With the parallel discovery of restriction enzymes in the early 1970s and pioneering work by Paul Berg, Stanley Cohen, and Herbert Boyer, recombinant DNA technology became a fact, enabling the manipulation of DNA, including viral genomes, to insert therapeutic genes that could be efficiently transduced (Cf. Chap. 1). Retroviral vectors were further optimized and became the workhorse for the transfer of therapeutic genes to correct disease phenotypes in vitro. By the mid-1980s, it was demonstrated that T cells from SCID patients lacking the enzyme adenosine deaminase (ADA) could be restored after retroviral vector-mediated delivery of genes encoding ADA. These findings formed the basis of the first human gene therapy clinical trial for ADA-SCID on September 14, 1990, at the National Institute of Health (NIH). The first patient included was Ashanti DeSilva, at the age of four. The medical team isolated the patient's T lymphocytes through apheresis, exposed these cells ex vivo to a genetically engineered live nonvirulent retrovirus carrying the normal ADA gene, and transfused these genetically modified T cells back into the patient's bloodstream. The treatment was successful in the sense that no adverse events were observed, and the low number of T cells that were transduced continued to express the recombinant transgene for over 12 years (Blaese et al. 1995; Muul et al. 2003), but treated patients still relied on enzyme replacement therapy. Now, 30 years later, over 3000 clinical trials with gene therapy were initiated, resulting in 18 gene therapies approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA).

In this section, we discuss the current state of gene therapy and the gene therapy medicinal products on the market, focusing on in vivo gene transfer. We will discuss how gene therapy can be applied and the various methods of gene transfer, including synthetic and viral vectors. Finally, we highlight the diseases currently subjected to gene therapy and touch upon the regulation of gene therapy products.

Gene Therapy: Definitions and Ways of Application

The definition of gene therapy continues to evolve to keep up with the ongoing technological advances. As a result, older definitions have become obsolete, and others are too broadly defined. Take, for example, the definition as applied by the European Medicines Agency: "gene therapy medicines contain genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting 'recombinant' genes into the body, usually to treat a variety of diseases, including genetic disorders, cancer or long-term diseases." Strictly speaking, any modification applied to the genome that does not involve the insertion of recombinant DNA is not considered gene therapy. With the rise of CRISPR-Cas (clustered regularly interspaced short palindromic repeats) gene editing, base editing, and prime editing (see section "Designer Nucleases for Gene Editing") that can introduce modifications in the human genome without the use of recombinant DNA, this definition falls short. Conversely, the FDA uses a much broader definition: "Human gene therapy seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use." FDA generally considers human gene therapy products to include all products that mediate their effects by transcription or translation of transferred genetic material, or by specifically altering host (human) genetic. According to this definition, any intervention that leads to altered gene expression would be considered a gene therapy, including those that do not directly change the genetic makeup of a cell such as gene silencing using RNA interference or the delivery of therapeutic mRNA. To bring some clarity and to draw a clear line as to where gene therapy ends and other nucleic acid therapies start (cf. Chap. 13), we propose the following definition: gene therapy is any intervention that leads to deliberate and long-lasting genomic alteration(s) or episomal expression of recombinant DNA with the aim to treat or cure a disease. This definition excludes interventions that affect gene expression at the level of transcription or translation, such as siRNA therapy, mRNA therapy, and antisense ON therapy, which are often transient and require frequently repeated dosing (see Fig. 14.13).

Gene therapy can be applied in three different ways: gene augmentation or addition, gene correction, and gene knockout.

Gene Augmentation

With **gene augmentation or addition**, an intact copy of a malfunctioning, disease-causing gene is inserted into the genome of patient cells (Fig. 14.14). This approach can only be used if the disease is caused by a "loss-of-function" gene mutation. These are mutations in a gene that causes the encoded protein to (partially) lose its function, which in turn causes disease. Loss-of-function mutations are typically recessive, meaning that both copies of the autosomal gene have defects. The addition of an intact copy of the gene under the control of a strong promoter thereby restores this func-

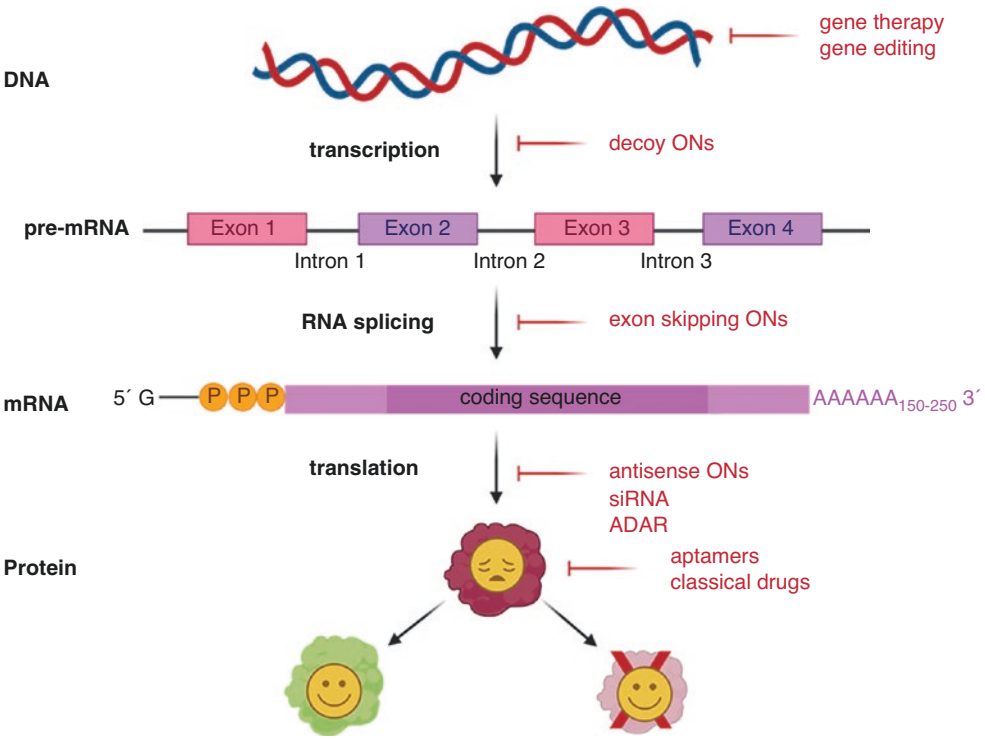

Fig. 14.13 Overview of different modes of intervention of therapeutic nucleic acids. Gene therapy and gene editing act directly on the genomic DNA and will be discussed in this chapter. Other interventions with nucleic acids, including synthetic oligonucleotides (ONs), small interfering RNA (siRNA), adenosine deaminase acting on RNA (ADAR), and mRNA that act at the level of transcription, RNA-splicing or translation are discussed in Chap. 13. Created with BioRender.com

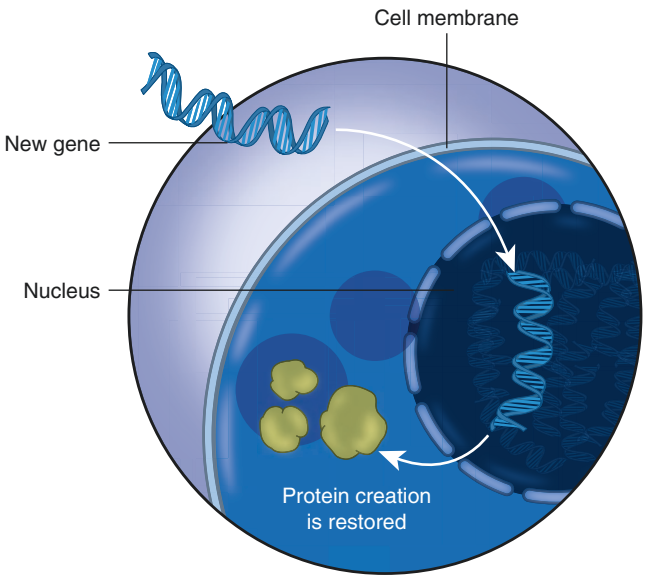

Fig. 14.14 The principles of gene addition to restoring loss-of-function mutations by inserting an intact copy of a gene (randomly) into the genomes of affected cells

tion. Insertion of the therapeutic transgene into the genome can be random or targeted to a specific region within the genome. Both viral and synthetic vectors can be used to deliver such gene constructs into affected cells (see section "Delivery Systems").

Gene Knock Out

A gene mutation might sometimes lead to a mutant protein that acquires a new, disease-causing function. Such mutations are referred to as "gain-of-function" mutations and are often dominant. Take, for example, the hereditary form of transthyretin amyloidosis (ATTR), which occurs when mutations in the *TTR* gene for transthyretin lead to instability of the tetrameric protein and formation of aggregates and fibrils that damage cells, leading to clinical symptoms. Such a gain-of-function mutation can only be treated if the mutated protein production is halted. This can be done by **gene knock out** in which a targeted disruption of the mutated gene (but not the unaffected allele) is introduced. CRISPR-Cas is a technology often used for creating a targeted gene knock out (see Sect. "CRISPR-Cas9" below).

Gene Correction

Gene correction aims to alter the disease-causing gene mutation. These corrections can be small (point mutations) or require the insertion of large pieces of DNA in case the mutation involves partial deletion of a gene. Gene editing systems such as Zinc-finger nucleases, TALENs (Transcription activator-like effector nucleases), CRISPR-Cas, base editors, or prime editors (see section "Designer Nucleases for Gene Editing" and Cf. Chap. 9) have been developed for this purpose. As such corrections are often very precise and include the use of molecular scissors to cut

open the genomic DNA at precise locations, gene correction is sometimes referred to as gene surgery.

Gene editing tools can be categorized based on those that introduce double-strand cuts, those that only cut one strand of the DNA or those that do not cut at all.

Somatic Versus Germline

Gene therapy can be applied to modify individual cells in the body. This is called somatic gene therapy and only affects the patient being treated. The corrected traits will not be inherited by potential offspring. Conversely, germline gene therapy aims to modify the germ cells (sperm cells and/or egg cells) to correct genetic mutations in the germline. If such modified germ cells are being used for reproduction, this will lead to offspring in which the gene correction is carried by all cells of the body, including the germline cells. Such corrections will therefore be passed down from generation to generation. Since gene therapy is a relatively new form of therapy, we do not yet know the long-term side effects of this, and as such, germline modifications would be unethical to perform at this stage. Many scientists, therefore, call for a global moratorium on germline gene editing (Lander et al. 2019).

Ex Vivo Versus In Vivo

Ex vivo gene therapy involves the genetic modification of cells outside of the body and their subsequent transplantation back into patients (Fig. 14.15). The advantage of this approach is that there is no patient exposure to the gene transfer vector, which can sometimes be harmful. Furthermore, the correctly modified target cells can be selected, expanded, and, if desired, differentiated before being transferred back to the patient to improve efficacy and safety. The limitation of such an ex vivo approach is that it

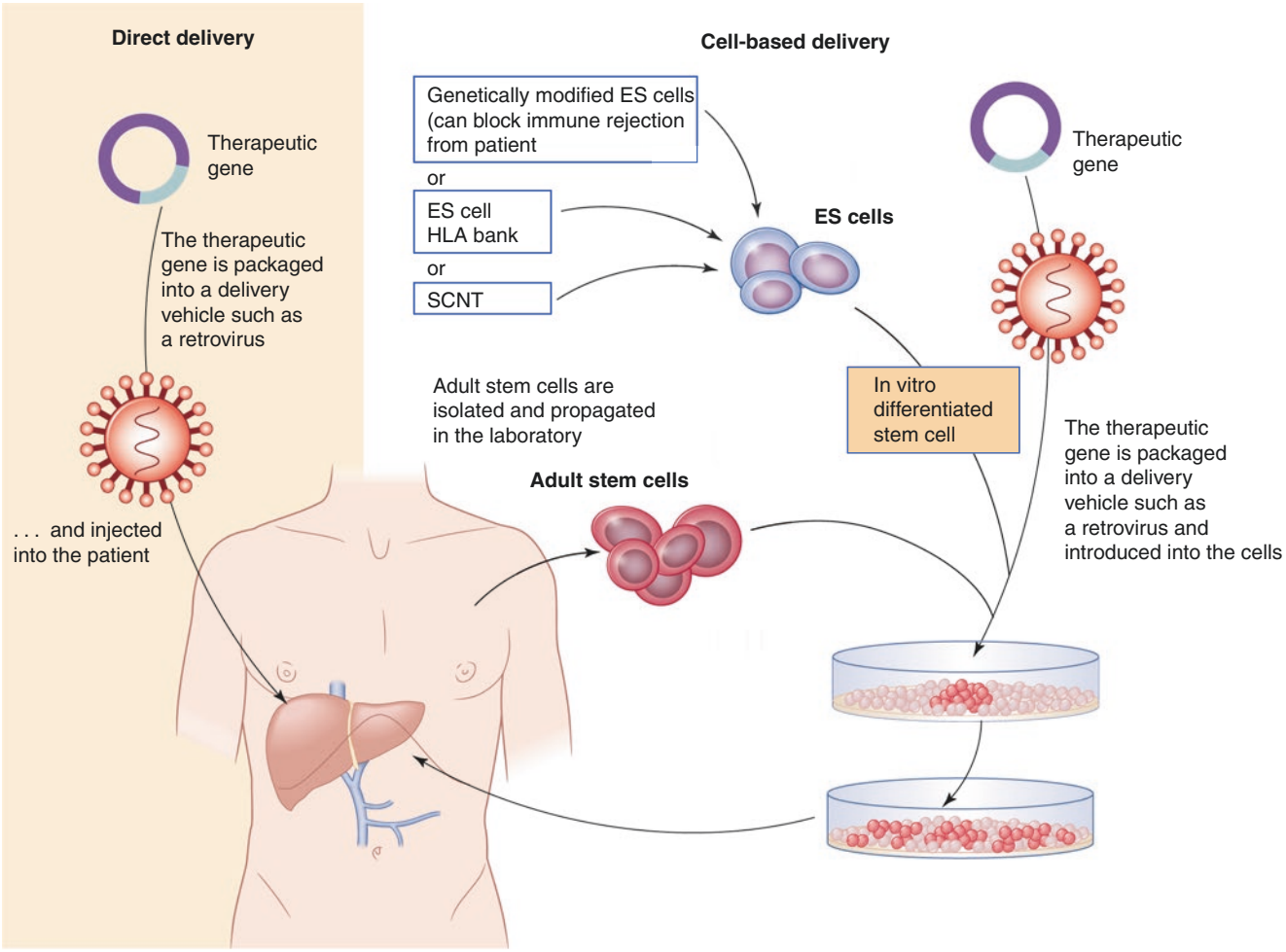

Fig. 14.15 Methods of administration of gene therapy vectors. In vivo gene transfer involves direct administration of the vector in the tissue of interest. Ex vivo gene transfer requires the collection of cellular targets from the patient. The cells are treated in culture with the vector. Cells expressing the therapeutic transgene are harvested and given back to the patient. ES: Embryonic Stem (cell). SCNT: somatic cell nuclear transfer (strategy). From Zwaka 2006; with permission to reprint

can only be applied to cells easily isolated from the human body (e.g., hematopoietic stem cells).

In vivo, gene therapy uses gene transfer vectors locally (e.g., into the eye or muscle) or systemically (via the bloodstream) to reach distant organs or tissues in the patient. Both viral and synthetic vectors have been developed for this purpose, which will be detailed in the section below. The advantage of this approach is that it avoids cumbersome cell isolations and manipulations in the laboratory and can, in principle, be applied by a single injection. The downside is the relatively low gene transfer efficiencies and potential vector-related (immune)toxicities to which the patients are exposed.

Designer Nucleases for Gene Editing

Designer nucleases are engineered nucleases that can be targeted to unique sequences in the human genome to introduce a double-strand DNA cut (Merkert and Martin 2016). As a result of this genomic DNA damage, the endogenous DNA repair system is activated to repair these cuts and ligate the ends of the fragmented DNA. Several different DNA repair pathways can be employed by the cell, which include nonhomologous end-joining (NHEJ), microhomology-mediated end-joining (MMEJ) and homology-directed repair (HDR)(Fig. 14.16).

With NHEJ, the ends of the damaged DNA recruit a protein complex that polishes the DNA: it removes damaged or mismatched nucleotides and randomly fills in nucleotides before the ends are ligated back to each other. As a consequence, NHEJ often leads to small insertions or deletions (indels) at the cut site. If this cut is inside a gene, such indels often lead to a frameshift in the coding sequence, and loss of protein function. It can therefore be used to knock out genes with gain-of-function mutations. MMEJ uses microhomology regions that may be present after end resection of the DNA ends, leading to the base pairing of the single-strand DNA overlaps and removal of the remaining pieces of ssDNA. This leads to deletions at the cut site. Both NHEJ and MMEJ are active throughout the cell cycle and thus active in both dividing and nondividing cells. However, these pathways do not allow precise editing as they tend to lead to errors.

For precise editing, the homology-directed repair is preferred. HDR is mostly active in cells in the G2 and S phases

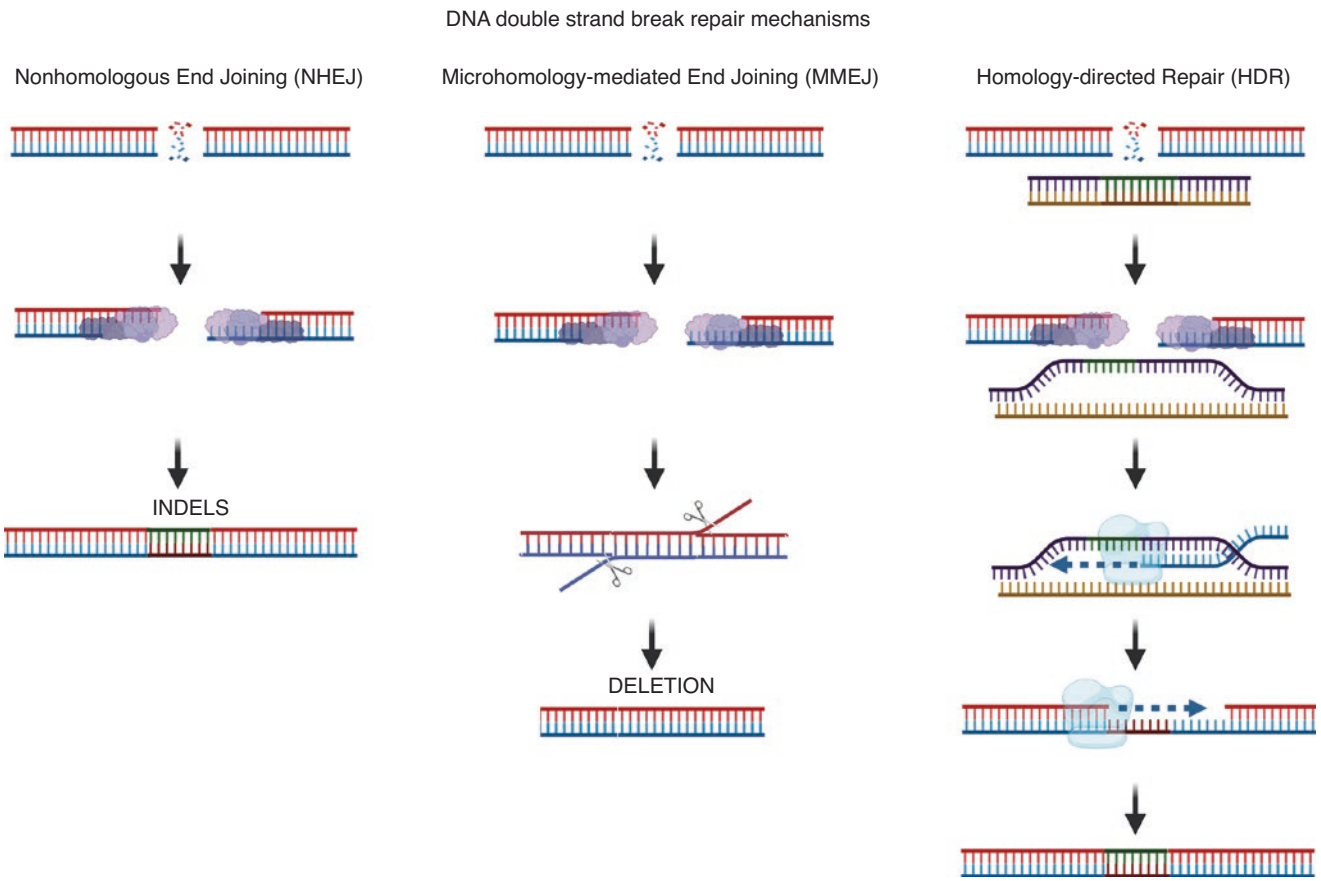

Fig. 14.16 Different pathways for DNA double-strand break repair. For details, see the text. Created with BioRender.com

and is meant to repair broken DNA strands prior to mitosis, making use of the homologous DNA from a sister chromatid to guide the repair. In a similar fashion, DNA double-strand break repair can be guided by providing an exogenous DNA template during repair. This HDR template can be single or double-stranded DNA with on the 5' and 3' ends sequences that are homologous to the sequences surrounding the double-strand cut. The length of such homology arms can vary from only 30 bases up to a few hundred or even thousand bases, depending on the overall size of the DNA to be inserted at the double-strand cut size. The exact working mechanism of HDR is still under debate, but it is generally believed that it involves 5′ to 3′ resection of the DNA ends at the double-strand cut site, after which the 3′ single-strand DNA overlap can invade the homologous DNA duplex of the HDR template to initiate the recombination repair process (Fig. 14.16). Compared to NHEJ and MMEJ, HDR is much less error-prone and often enables precise gene editing. Using single-stranded synthetic oligonucleotides of only 60 bases, specific point mutations can be introduced at a precise location within the genome (Shy et al. 2022). This technology offers great potential to correct debilitating diseases caused by a single point mutation in a single gene, such as sickle cell anemia, β-thalassemia, transthyretin amyloidosis (ATTR), or certain genetic eye diseases.

The first designer nucleases to be engineered were the zinc-finger nucleases (ZFNs), followed by transcription activator-like effector nucleases (TALEN), and more recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and its CRISPR associated Cas protein (CRISPR/Cas) (Fig. 14.17) (LaFountaine et al. 2015).

They consist of an engineered restriction endonuclease, often *FokI*, fused to 3-6 zinc-finger proteins, each recognizing a specific three-base pair sequence. *FokI* from the bacterium *Flavobacterium okeanokoites,* only cleaves DNA when it is bound to its recognition sequence GGATG and when it can form dimers. By directing *FokI* monomers to specific sequences on both strands of the DNA, *FokI* monomers can be positioned to form dimers and introduce a double-strand cut in the genomic DNA (Fig. 14.17). Although initially popular, the need for generating complex fusion proteins, which is often time-consuming, and the CRISPR-Cas technology's emergence has made this gene editing technique less popular.

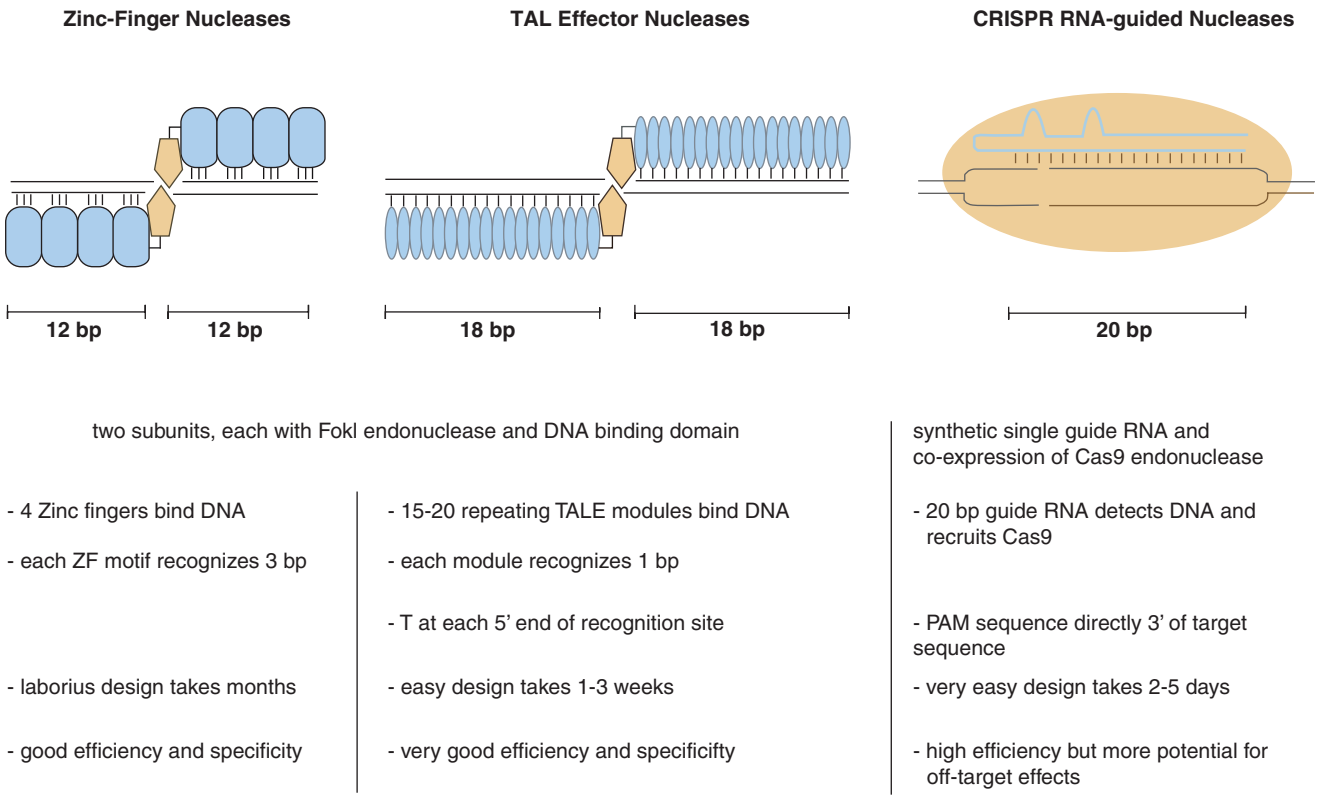

Fig. 14.17 Three types of designer nucleases for targeted genome editing. Zinc-finger nucleases and TAL effector nucleases make use of modular DNA-binding proteins to target the FokI nuclease to a specific sequence, whereas CRISPR-Cas uses an associated RNA to target the Cas nuclease to a specific sequence

TALEN

Similar to ZFNs, the transcription activator-like effector nucleases (TALEN) exploit customizable DNA-binding proteins fused to a FokI nuclease. The TALE DNA-binding domains were identified as secreted proteins from the *Xanthomonas spp.* bacteria and consist of highly conversed 33–35 amino acid repeats with two amino acid repeat-variable diresidues (RVD), which dictates individual nucleotide specificity. Assembling repeats into TALE arrays flanked by essential TALE-derived N and C-terminal domains fused to FokI repurposes the system for genome editing. As a result, TALENs are very precise with low off-target editing and are still popular in gene therapy applications. For example, several companies have used this technology for engineering chimeric antigen receptor (CAR) T cells for cancer immunotherapy.

CRISPR-Cas9

CRISPR, an acronym for Clustered Regularly Interspaced Short Palindromic Repeats, and its associated proteins (Cas) are part of the bacterial adaptive immune system to shield invading viruses and which has been repurposed for gene editing in human cells. The Cas9 gene editing system consists of a Cas9 endonuclease interacting with a trans-activating RNA (tracrRNA), which is directed to a target sequence by ~ 20 nt complementary sequences in the CRISPR RNA (crRNA) and flanked by the 3′ protospacer adjacent motif (PAM). The crRNA and tracrRNA can be joined by a tetraloop to form a single guide RNA (sgRNA) (Fig. 14.18). When a sgRNA:Cas9 ribonucleoprotein binds its target sequence in the presence of a flanking PAM sequence, the Cas9 protein will introduce a double-strand break (DSB). If a PAM sequence is lacking, the Cas9 will not cut. Cas9 proteins from different bacteria recognize different PAM sequences. The most frequently used are Cas9 from *Streptococcus pyogenes* (spCas9; PAM 5′-NGG-3′, where N is any nucleobase) and Cas9 from *Staphylococcus aureus* (saCas9; PAM 5'-NNGRRT-3', where R is a purine), but many different natural and engineered variants of these Cas nucleases exist. For example, Cas12a, previously known as Cpf1 from *Acidoaminococcus sp.*, does not require a tracrRNA but only a crRNA. After binding its target sequence and identifying its PAM (5'-YTN-3', where Y is a pyrimidine) Cas12a introduces a sticky-end cut, with 4-5 nucleotides overhang. The advantage of CRISPR-Cas for gene editing over the use of ZFN or TALEN is the ease by which the system can be adapted to target a specific sequence in the genome. Whereas for TALEN and ZFN, it is required to engineer a new targeted nuclease for each target sequence, which can take weeks to months as it requires elaborate recombinant protein expression and purification, the target specificity of Cas9 and Cas12a is completely determined by the associated guide RNA, which can be easily synthesized. A downside of CRISPR-Cas gene editing system is its relatively high base mismatch tolerance, meaning that it can introduce DSB at places in the genome with near-identical 20-nt sequences compared to the intended target sequence (as long as a PAM sequence is present). It has been reported that off-target events can occur with as many as 3-5 base pair mismatches. This could introduce unintended mutations in the genome at potentially harmful places. For that reason, Cas9 proteins have been engineered to increase specificity. For example, spCas9-HF1 has very low levels of off-target

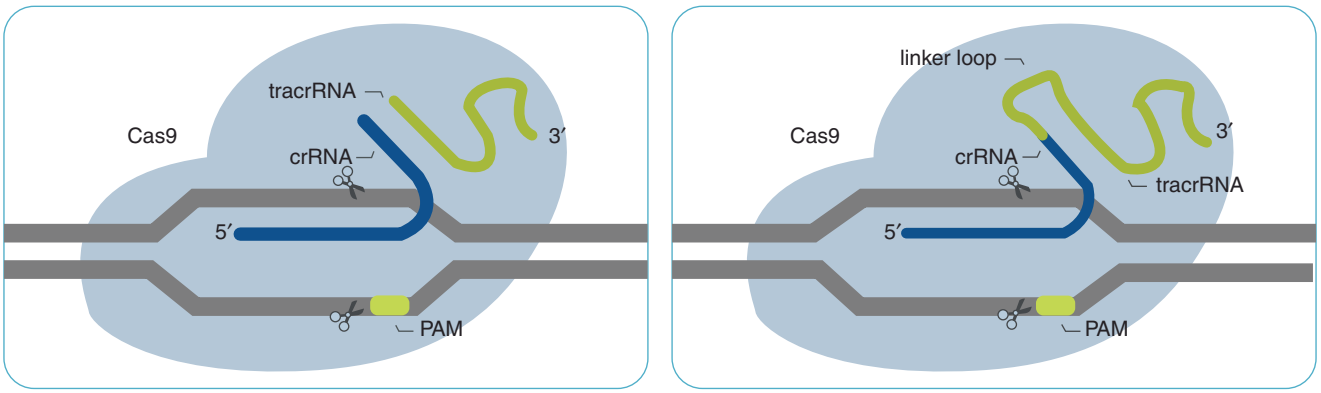

Fig. 14.18 The Cas9 gene editing system consists of a Cas9 nuclease with an associated trans-activating RNA (tracrRNA), which is directed to a target sequence by ~ 20 nt complementary sequences in the CRISPR RNA (crRNA) and flanked by the 3′ protospacer adjacent motif (PAM) (left panel). Scissors indicate the location of the double-strand breaks introduced by Cas9. The tracrRNA and crRNA can also be joined by a linker loop to form a single guide RNA (sgRNA; right panel)

effects, albeit at the cost of lower on-target cutting efficiency than wild-type spCas9 (Kleinstiver et al. 2016).

Despite these improved Cas9 nucleases, introducing DSB still imposes a risk during therapeutic gene editing. Besides introducing unintended mutations at off-target sites, generating DSB in genomic DNA can also lead to large deletions and chromosomal translocations. As a result, Cas mutants have been generated that can still be targeted to specific sequences in the genome but are devoid of nuclease activity. These Cas mutants form the basis of an entirely new class of gene editing tools without DSB. The most frequently used will be discussed below.

Base Editors

DNA Base editors allow the introduction of point mutations in the DNA without generating DSB. They consist of two domains: a catalytically "dead" Cas9 enzyme (dCas9) or a nickase (nCas9), fused to a single-stranded DNA modifying enzyme for targeted nucleotide alteration (Fig. 14.19). Two classes of DNA base editors have been described: cytosine base editors (CBE) and adenine base editors (ABE). Cytosine deamination generates uracil, which base pairs as thymidine in DNA and thus converts C:G into A:T base pairs. Conversely, ABEs convert A:T into G:C base pairs. Collectively, all four transition mutations (C→T, T→C, A→G, and G→A) can be installed. Besides these two major base editors, new variants are being developed, including those that introduce a base transversion (C→G), expanding the scope of disease-causing point mutations that can be edited (Kurt et al. 2021). The distance at which the base editors operate within the protospacer (i.e., the target sequence to which the base editor is bound)

is called the editing window and is dependent on the type of BE being used. For CBE, the editing window spans positions 4-8 of the protospacer, and for ABE positions 4-7 (ABE7.10) or 8-10 (ABE6.3, ABE7.8, or ABE7.9).

Conversion of multiple nucleobases within the editing window is possible and may lead to undesired edits, called bystander edits. Adapted from Antoniou et al. (2021).

Prime Editors

While BEs can, in principle, correct the majority of pathogenic point mutations, they cannot perform all possible single-nucleotide conversions and also cannot mediate targeted insertions or deletions. The solution for this is prime editors (PEs) developed by Anzalone et al. (2019). A PE consists of a reverse transcriptase fused to a nCas9 nickase (Fig. 14.20). In combination with an engineered prime editing guide (pegRNA), this construct is directed to a target sequence in the genome, which nicks the noncoding DNA

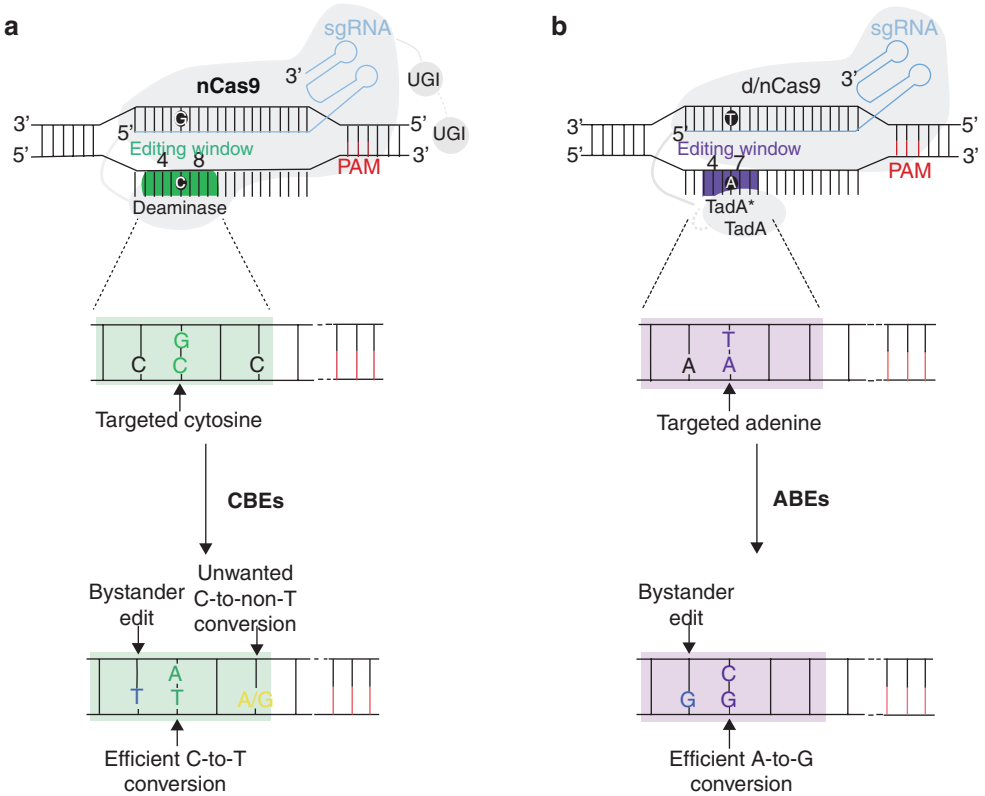

Fig. 14.19 Cytosine and Adenine base editors. A. Cytosine base editors (CBE) consist of a nCas9 fused to a cytosine deaminase and a uracil glycosylase inhibitor and convert C:G into T:A base pairs. The editing window within the protospacer is illustrated in green. B. Adenine base editors (ABE) consist of a dCas9 fused to an engineered adenine deaminase from *E. coli* (TadA) and convert A:T into G:C base pairs. The editing window is illustrated in purple

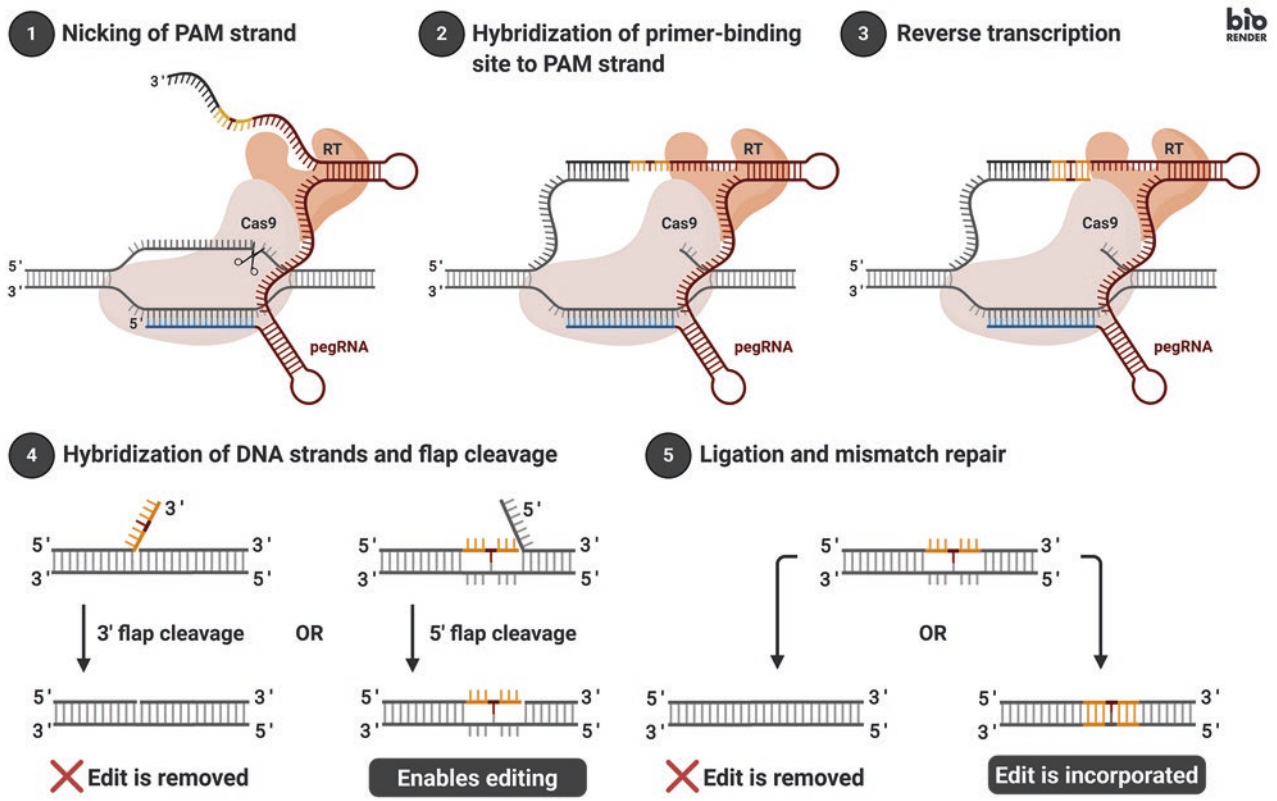

Fig. 14.20 Mechanism of prime editing (PE). Once bound to its cognate targeting sequence, the PE introduces a nick (1), releasing the 3' end of the PAM strand, which can hybridize with the pegRNA extension (2) to initiate reverse transcription (3). The formed branched DNA structure either contains a 3' flap containing the edited sequence or a 5' flap containing the original, unedited sequence, which will be removed by exonucleases, after which the nick is ligated (4). In case of incorporation of the edited strand, a heteroduplex is formed that will be repaired by intrinsic mismatch DNA repair pathways (5)—figure copied from Wikipedia

strand. Hybridization of the 3' end of this DNA strand with the pegRNA initiates reverse transcription using the pegRNA extension as a template. This leads to a branched DNA intermediate with either a 3' flap (containing the edited sequence) or 5' flap (containing the original, unedited sequence) sticking out. Exonucleases remove these flaps, yielding in 50% of the cases a heteroduplex DNA composed of one edited and one unedited strand. The intrinsic mismatch DNA repair pathway can then repair this mismatch with two possible outcomes: the edited strand is copied to the complementary strand, or the unedited strand is restored.

PE is a very flexible gene editing tool that can introduce base transitions, transversions, and deletions (up to 80 bp) or insertions (up to 60 bp). However, it is less suitable for inserting larger pieces of DNA. The downside of PEs is their low efficiency of editing, which might contribute to the edit's various outcomes and instability of the rather large pegRNA. Further research is needed to make this method of gene editing more efficient.

Delivery

For gene therapy to be effective, the transgene construct or gene editing tools must be delivered to diseased cells in the body. For inherited diseases, this would, in principle, be all somatic cells in the body, but this will technically not be feasible. Hence, delivery strategies focus on targeting cells in vivo or ex vivo that are mostly affected by the genetic disease. In the section below, we discuss the various gene delivery vectors that have been developed for gene therapy applications. This includes viral vectors, synthetic vectors, and vectors derived from extracellular vesicles.

Viral Vectors

Viruses have evolved to introduce their genetic material into the host cell efficiently. These properties can create a vehicle to deliver genes for expression into cells. This is called a viral vector. A viral vector differs from the native counterpart in that it cannot replicate and is less pathogenic: genes involved in viral replication and pathogenicity are removed

to generate a safe vehicle with enough space to include transgene cassettes. The most commonly used viral vector systems for gene therapy purposes are adeno-associated viral (AAV) and retroviral vectors.

To produce functional viral vector particles, the genes required for the structural viral proteins need to be provided *in trans* with the transfer vector, i.e., a piece of DNA, often plasmid DNA containing the therapeutic gene construct. The genes encoding the viral proteins for replication and packaging of the transfer vector can be placed on plasmids and transfected into producer cells (Fig. 14.21). The transfer vector retains minimal sequences required for stability, replication, or integration. These elements include the terminal sequences called inverted terminal repeats (ITRs) for AAV vectors and long-terminal repeats (LTRs) for gammaretroviral vectors. Additional elements, such as cis-acting elements, e.g., a packaging signal (ψ), may be essential for the efficient incorporation of the transfer vector into the viral particle. Retroviral vectors, besides the packaging signal, may also require truncated leader regions for efficient packaging. Other virus-derived sequences may be included to enhance delivery to the nucleus, such as the central polypurine tract (cPPT) in lentiviral vectors (a subtype of retroviral vectors) (Zennou et al. 2000), or elements to enhance expression of the transgene, such as the commonly incorporated Woodchuck hepatitis posttranslational regulatory element (WPRE). This will be discussed in more detail below. Retroviruses, including gammaretroviral vectors, lentiviral vectors, more recently alpharetroviral vectors, and AAV, are the most extensively studied for ex vivo and in vivo use in monogenic diseases. Their characteristics are summarized in Table 14.9.

Retrovirus

Biology

Retroviruses are membrane-enveloped RNA viruses containing two copies of a positive single-stranded RNA genome (Fig. 14.22). The retroviral vector systems that are commonly used in clinical applications for monogenic diseases are derived from gammaretroviruses and lentiviruses. More recently, alpharetroviral vectors have also been developed. Retroviruses are ~80–145 nm in diameter and have a genome size of about 7–10 kb, composed of a group-specific antigen gene (*gag*), which codes for core and structural proteins of the virus; polymerase (*pol*) gene, which codes for reverse transcriptase, protease, and integrase; and envelope (*env*) gene encoding the retroviral envelope glycoproteins. The long-terminal repeats (LTRs) control the expression of viral genes, hence act as enhancer-promoter. The packaging signal (ψ) helps efficient incorporation of the viral positive-strand RNA into the virus particle before budding off the cell membrane (Verma 1990).

After viral binding and introducing the viral RNA into the host cell, reverse transcriptase, which has both polymerase and RNase activity, converts the viral RNA to linear double-stranded DNA that integrates into the host genome with the help of the viral integrase. The integrated virus sequence, the provirus, will later undergo transcription and translation to produce viral genomic RNA encoding viral proteins. Virus particles then assemble in the cytoplasm and bud from the host cell to infect other cells.

Suitability of Retroviruses as Vectors for Gene Transfer

To generate replication-deficient retroviral vectors, the sequences encoding the virion proteins (gag, pol, and env) responsible for the viral replication and pathogenicity are removed if redundant or placed in a split packaging system to produce the vector particles. The space that is created by deleting viral genes can be used to insert a transgene cassette. The transgene can be controlled by the native LTRs, which have intrinsic enhancer/promoter activity, or by including exogenous enhancer-promoter sequences. For the production of retroviral particles, the vector containing plasmid is introduced into packaging cell lines, mostly HEK293 cells, to produce the retroviral vectors.

Retroviral vectors have several features for gene transfer applications that are important to consider (Table 14.9). They can accommodate transgene cassettes of 8 kb and integrate them into the host genome. Therefore, retroviral vectors can provide stable, long-term transgene expression in dividing cells with low immunogenic potential, particularly because these vectors are mostly used for ex vivo applications. However, there are several disadvantages to these vectors. Gammaretroviruses and alpharetroviruses cannot transduce nondividing cells, but lentiviral vectors can overcome this hurdle, by transporting the preintegration complex (PIC) through the nuclear pores by an active, energy-dependent process (Bukrinsky et al. 1992), as has been shown for transduction of neurons, hepatocytes and hematopoietic stem cells (HSCs). Current methods of viral vector production generate preparations in which the virus titer is sufficient (1×10^5–1×10^7 active viral vector particles/mL) but can generally only be used for a limited number of patients. Retroviruses are also inactivated by elements of the complement system and rapidly removed from the systemic circulation in response to cellular proteins incorporated in the viral envelope during the budding process. Therefore, there are limited clinical trials with retroviral vectors for direct in vivo gene therapy.

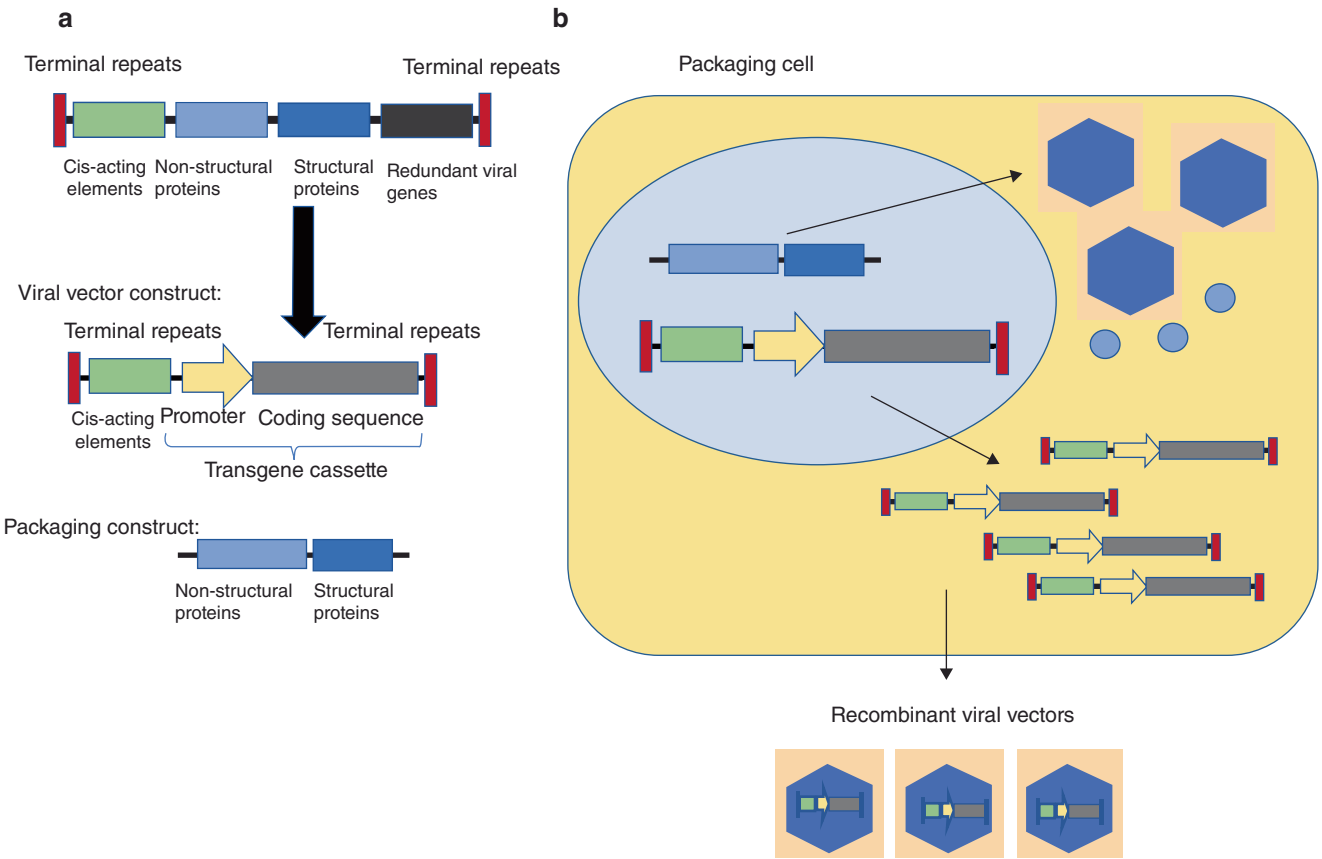

Fig. 14.21 Schematic representation of a virus vector system for gene transfer. (**a**) Simplified overview of a viral genome (top), that forms the basis for the packaging construct (containing gene sequences of structural proteins and nonstructural proteins involved in replication), and the vector transfer construct (containing the transgene cassette). The viral genome contains genes involved in replication, proteins for the virion structure, and the pathogenicity of the virus, which can be removed from the viral vector construct. It is flanked by the terminal sequences (ITRs for AAV or LTRs for retroviral vectors) and cis-acting elements, such as signals for viral packaging. The packaging construct contains only genes that encode nonstructural and structural proteins. The viral vector construct contains the terminal sequences (ITRs or LTRs), required cis-acting elements, and the transgene cassette with promoter and coding sequences (therapeutic gene). The transgene cassette includes a complementary polyadenylation (polyA) termination signal in an AAV vector. In retroviral vectors, this signal is contained in the LTR (**b**). The packaging and vector constructs are brought into the packaging cell by transient transfection, by infection with a helper virus, or by generating stable cell lines applicable to the virus vector system used. The packaging construct expresses replication-related proteins and viral structural proteins. The vector sequences are produced and encapsidated to generate the recombinant viral vector particles

A limitation of retrovirus-based gene therapy is that gammaretrovirus tethers to the transcription start sites and promoters, inserting the genetic cargo semi-randomly into the host genome (Deichmann et al. 2007, 2011; Schwarzwaelder et al. 2007). This can lead to genotoxic events through multiple mechanisms. The vector integration could cause insertional mutagenesis, disrupt and alter the gene expression of a gene close to the integration site. In addition, gammaretroviral vectors have a bias to integrate into proto-oncogenes (Cattoglio et al. 2007), which could cause insertional oncogenesis. Indeed, this serious adverse event was observed in clinical trials, which pushed the field to create safer retroviral vector designs. For instance, modifications have been made to delete part of the U3 region in the 3' LTR to create a self-inactivating (SIN) configuration. After reverse transcription, the 3' U3 region is copied to the 5' LTR creating two LTRs lacking enhancer/promoter activity. This modification enables the use of internal expression cassettes with physio-

Table 14.9 Characteristics of viral vectors for gene transfer[a]

	Gammaretrovirus	Lentivirus	Alpharetrovirus	Adenovirus	Adeno-associated virus
Genetic material	RNA	RNA	RNA	dsDNA	ssDNA
Genome size	7–11 kb	8 kb	9 kb	26–45 kb	4.7 kb
Cloning capacity	8 kb	8 kb	5.8–8.8 kb	7[b]–35[c] kb	<5 kb
Genome forms	Integrated	Integrated	Integrated	Episomal	Stable/episomal
Diameter	100–145 nm	80–120 nm	80–100 nm	80–100 nm	20–12 nm
Tropism	Dividing cells only	Broad, dividing and nondividing cells	Dividing	Broad, dividing and nondividing cells	Broad, not suitable for hematopoietic cells
Virus Protein Expression	No	Yes/no	No	Yes[b]/no[c]	No
Delivery method	Ex vivo	Ex vivo	Ex vivo	In vivo	In vivo
Typical yield (viral particle/ml)	$<10^8$	$<10^7$	$<10^7$	$<10^{14}$	$<10^{11}$
Pre-existing immunity	Unlikely	Perhaps, post-entry	Unlikely	Yes	Yes
Immunogenicity	Low	Low	Low	High	Moderate
Potential pathogenicity	Low	High	Low	Low	None
Applications	HSC gene therapy, cellular immunotherapy	HSC gene therapy, cellular immunotherapy	HSC gene therapy; cellular immunotherapy	Oncology	Inherited diseases, postmitotic tissues
Development phase	Clinical stage	Clinical stage	Preclinical stags	Clinical	Clinical
Safely	Insertional mutagenesis	Insertional mutagenesis	Insertional mutagenesis	Potent inflammatory response	Insertional mutagenesis long-term risk not clear. Risk of hepatotoxicity
Physical stability	Poor	Poor	Poor	High	High

[a] Information compiled from references (Edelstein et al. 2004; Weber and Fussenegger 2006)
[b] First-generation, replication-defective adenovirus
[c] Helper-dependent adenovirus

logical or cell type specific promoters reducing the risk of genotoxicity (insertional mutagenesis) and phenotoxicity (ectopic transgene expression) by restricting the expression level to certain cell types or tissues.

Gammaretrovirus

Biology

Gammaretroviruses, such as Moloney murine leukemia virus (MoMLV), were the first retroviral vectors used for clinical application. Gammaretroviruses were originally called oncoretroviruses, which caused tumors in mice. Cell lines derived from HEK293T are commonly used for the production of gammaretroviral vectors. These often generate producer cell lines using ecotropic or amphotropic MLV envelope protein for pseudotyping gammaretroviral vector particles. Pseudotyping means that different envelope glycoproteins are used instead of the wild-type glycoprotein. Amphotropic MLVs are subgroups that infect a broader range of cell types. The structural proteins and transfer vectors are commonly split over three expression plasmids. Vesicular stomatitis virus G (VSVg) glycoprotein can also be used to pseudotype gammaretroviral vectors, stabilizing particles and broadening their tropism. A drawback of using gammaretroviral vectors is that cell division is required for efficient transduction.

Clinical Use of Gammaretrovirus

Retroviral vectors are currently employed mostly for ex vivo gene therapy, such as hematopoietic stem cell (HSC) gene therapy or cellular immunotherapy applications. The gammaretrovirus MoMLV was the first viral vector used in the clinic for treating severe combined immunodeficiency ADA-SCID, a rare inherited disease in which the buildup of toxic deoxyadenosine due to lack of activity of the enzyme adenosine deaminase results in complete lack of T and B lymphocytes (Ferrua and Aiuti 2017). In addition, MoMLV-vector expressing recombinant *ADA* was used for ex vivo genetic modification of autologous peripheral blood lymphocytes (Ferrua and Aiuti 2017).

Other successful clinical trials employing gammaretroviral vectors were performed to treat a rare X-linked SCID (X-SCID) (Kohn and Kohn 2021). MoMLV-vectors expressing *IL2RG* cDNA (also known as common gamma chain) were used to transduce autologous HSCs isolated from

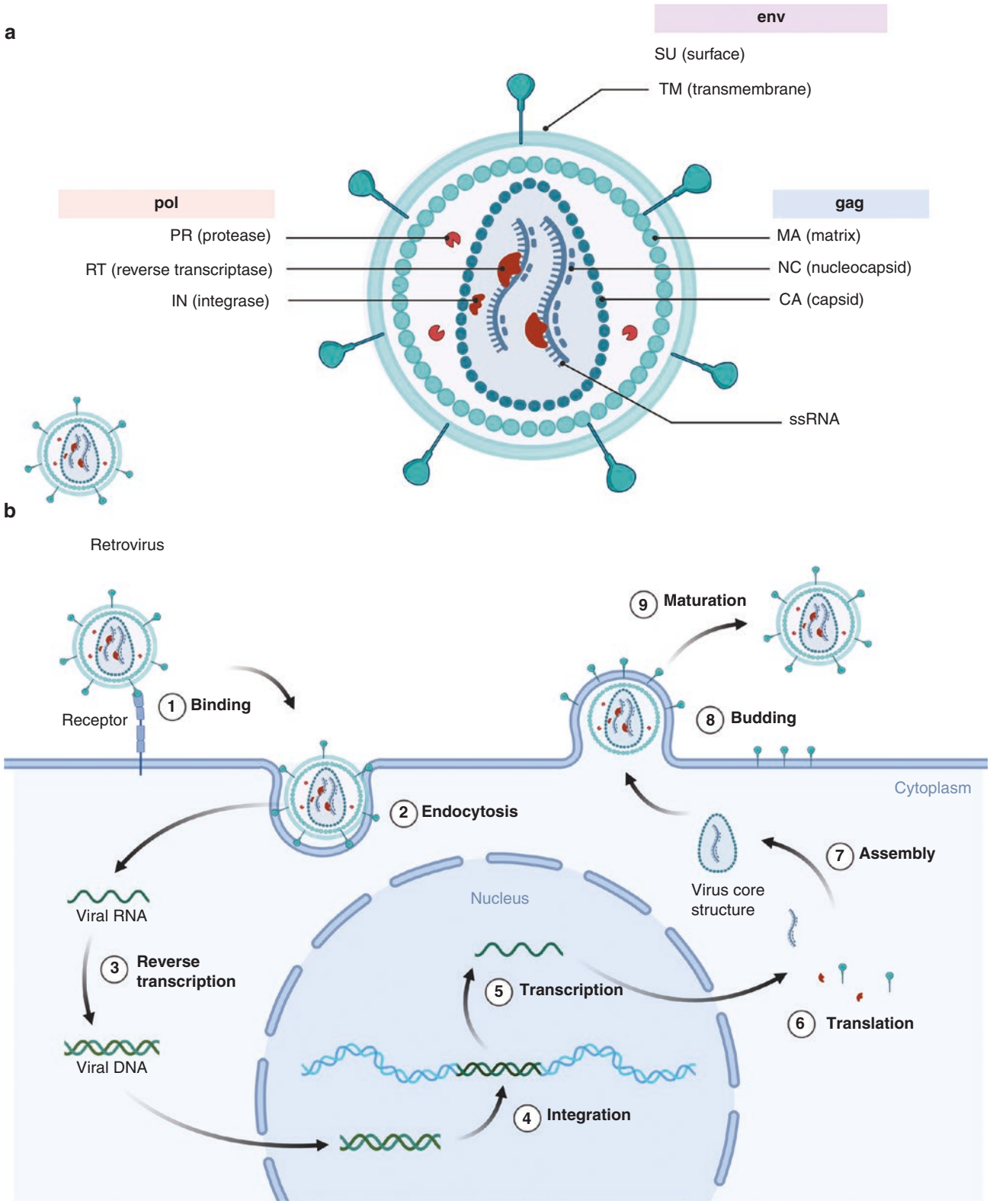

Fig. 14.22 The retrovirus. (**a**) Schematic cross-section of a retrovirus. (**b**) The retrovirus replication cycle. Retroviruses enter cells by receptor-mediated endocytosis (1,2). The viral RNA is reverse-transcribed into double-stranded DNA (3), which is then shuttled into the nucleus, which requires cell division and opening up of the nucleus in most retroviruses. For HIV-1, the viral RNA and core are shuttled into the nucleus, after which reverse transcription occurs (Dharan et al. 2020; Selyutina et al. 2020). Integrase catalyzes the double-stranded viral DNA in the nucleus to integrate into the host genomic DNA as provirus (4). The host cell RNA polymerase transcribes the viral RNA from the integrated provirus in the nucleus (5). Full-length and spliced viral RNA molecules are shuttled out of the nucleus and serve as templates for translation into viral proteins (6) that are cleaved by the protease to form the structural proteins of the virus particle and incorporate two full-length positive-strand RNA copies. The viral cores of the virus particles are assembled (7), and the viral particle buds from the cell membrane (8), after which virus maturation occurs (9). Created with Biorender.com

X-SCID patients without an HLA-identical sibling donor for ex vivo transduction. Subsequently, the genetically modified HSCs were transfused back to patients without preceding cytoreductive chemotherapy to reconstitute lymphocyte and natural killer cell lineages. These initial gene therapy trials enrolled 20 patients (Kohn and Kohn 2021) and showed restoration of immune function in 18 out of 20 patients, but some patients still required immunoglobulin infusions. However, 6 out of 20 patients developed T-cell leukemias due to insertional oncogenesis (Fischer and Hacein-Bey-Abina 2020; Kohn and Kohn 2021). This initiated the construction of self-inactivating gammaretroviral vectors (Kohn and Kohn 2021), which include weaker physiological promoters, such as elongation factor 1 alpha short promoter lacking enhancer sequences. In another trial for Wiskott-Aldrich syndrome, nine out of ten patients developed T-cell leukemia using a similar vector design with transgene transcription driven by the viral LTR (Ferrua and Aiuti 2017). On the other hand, a similar gammaretroviral vector backbone has been used in ADA-SCID, with a much lower genotoxicity profile (Tucci et al. 2022). This emphasizes that vector design and possibly disease background are important factors that determine the outcome in clinical trials.

In addition to safety-enhanced features that have been incorporated into gammaretroviral vectors, other virus-derived vector systems with an enhanced safety profile, such as lentiviral vectors and more recently developed alpharetroviral vector systems, are being exploited.

Lentivirus

Biology

Lentiviruses are retroviruses that can replicate in both dividing and nondividing cells. The biology of lentiviruses resembles that of gammaretroviruses. Apart from the genes *gag*, *pol*, and *env*, lentivirus has six accessory genes, such as *tat*, *rev*, *vpr*, *vpu*, *nef*, and *vif*, which regulate the synthesis and processing of viral RNA and other replicative functions.

Human immunodeficiency virus (HIV) is the most commonly studied lentivirus and has served as the backbone for a viral vector system commonly used for ex vivo applications. Wild-type HIV infects human helper T cells and macrophages. Apart from the genes *gag*, *pol*, and *env*, the accessory genes that are required for HIV replication and pathogenesis in vivo are not required for viral particle production and can all be removed to create sufficient space to incorporate transgene cassettes. HEK293T cells are most frequently used as packaging cells for lentiviral vector production.

Lentiviruses as Vectors for Gene Transfer Vehicles

One of the advantages of using lentiviral vectors is that they can efficiently transduce nondividing cells or terminally differentiated cells such as neurons, macrophages, muscle, and liver cells for in vivo gene therapy, as well as HSCs and immune cells, such as T cells, NK (natural killer) cells and macrophages for ex vivo gene therapy. These cell types are more effectively transduced with lentiviral vectors than using gammaretrovirus-based gene therapy systems. Previous studies have shown that when injected into the rodent brain, liver, muscle, or pancreatic islet cells, lentivirus promoted a sustained gene expression for over 6 months (Miyoshi et al. 1997). Lentiviruses have a typical integration site pattern, which is different from gammaretroviral vectors. The lentiviral LTRs and proteins that bind the integration complex largely determine the integration pattern by tethering the preintegration complex into highly expressed genes (Biffi et al. 2011). These properties reduce the genotoxicity potential of lentiviral vectors compared to gammaretroviral vectors that integrate into transcription start sites (TSS) and have a preference for proto-oncogene integration (Cattoglio et al. 2007; Moiani et al. 2013). The creation of self-inactivating lentiviral vectors has even further reduced the potential of genotoxicity because physiological promoters can drive transgene expression instead of the viral enhancer/promoter (Tucci et al. 2022). The development of the third-generation lentiviral vectors by dividing the plasmid system over four plasmids separating the transfer sequences from gag/pol, the envelope glycoprotein, and the *rev* gene has reduced the risk of generating replication-competent lentivirus (RCL) significantly. The third-generation self-inactivating lentiviral vectors system is the most commonly used as opposed to the earlier generations. As an exogenous envelope glycoprotein, VSVg is also commonly used for pseudotyping to improve tropism and stability. In addition, *rev* is provided in trans to increase lentiviral vector titers by binding to the rev response element sequence in the transfer vector and accumulating unspliced viral RNA in the cytoplasm (Fritz and Green 1996). The introduction of point mutations into the HIV integrase or the LTRs creates lentiviral vectors that are integration defective. This application can provide long-term expression in nondividing tissues, such as skeletal muscle, eye, and liver (Gurumoorthy et al. 2022). The use of integration defective lentiviral vectors obviously reduces the risk for genotoxicity.

Clinical Use of Lentiviral Vectors

HIV-derived lentiviral vectors have been used in clinical trials for multiple indications, including inherited diseases. For example, to treat HIV infection, patient CD4 T cells were genetically modified ex vivo by the lentiviral vector VRX496 (MacGregor 2001). The VRX496 vector contained an antisense sequence targeted to the HIV env gene to interfere with

HIV replication. Although no treatment-related severe adverse events were observed, no statistically significant anti-HIV effects were observed either (Manilla et al. 2005) (cf. the "Cell Therapy" part of this chapter). Lentiviral vectors have been mostly used in clinical trials for primary immune deficiencies (PIDs), such as X-SCID and Wiskott-Aldrich syndrome, and X-linked chronic granulomatous disease (CGD) (Tucci et al. 2022), other blood disorders (sickle cell disease/thalassemia) (Staal et al. 2019) and (neuro)metabolic disorders, such as metachromatic leukodystrophy (MLD), Hurler syndrome or Fabry disease (Chiesa and Bernardo 2022) using genetically modified HSCs. In PIDs and blood disorders, there is an inherent defect in the blood lineages and function development. In (neuro)metabolic disorders, the hematopoietic system may produce recombinant protein for cross-correction and deliver protein in the central nervous system (see also chapter below on monogenic diseases).

Alpharetroviral Vectors

Biology

Alpharetroviral vectors have been more recently developed in addition to gammaretroviral and lentiviral vectors. This system is based on the Rous sarcoma virus (RSV), which was originally isolated from chicken sarcoma cells. RSV contains the viral tyrosine kinase *v-Src* gene, which triggers uncontrolled growth in infected cells (Rubin 2011). The *v-Src* gene increases virulence of RSV. The RSV genome was used to design a split packaging system separating *gag/pro* and *pol* sequences from the transfer vector, with a transfer vector containing self-inactivating configuration and replacing the RSV envelope glycoprotein (Suerth et al. 2014). In this design, the packaging plasmids do not share any homology with the transfer vector, as opposed to the lentiviral vector systems, which do contain sequence overlap between transfer and packaging plasmids. In addition, the alpharetroviral vector leader region is *gag*-sequence free. The integration pattern of alpharetroviral vectors has a more random profile as opposed to gammaretroviral vectors and lentiviral vectors, and aberrant splicing has not been observed (Suerth et al. 2014), which may contribute to safety.

Retroviral Mechanisms of Genotoxicity

Retroviral vectors integrate into the genome and can therefore provide stable transgene expression. However, this could also lead to dysregulation of genes close to the integration site. Three main mechanisms contribute to gene dysregulation and potential genotoxicity. The most common is that the enhancer/promoter elements of the integrated vector could upregulate the expression of genes, potentially proto-oncogenes (Williams et al. 2022). Another mechanism of genotoxicity is related to splice donor and acceptor sites interfering with endogenous gene splicing, generating fusion or truncated transcripts. Finally, genotoxicity may be caused by read-through transcripts from retroviral vectors, and this may induce leukemia. Since the retroviral vector systems most commonly exploit the transcriptional termination signals in the 3' LTR, which are generally poor terminators, transcript read-through may occur. Transcript read-though may be reduced by including elements that improve transcriptional termination (Suerth et al. 2014; David and Doherty 2017).

Genotoxicity risks also depend on cell source selection. For example, lentiviral vector integration sites in postmitotic tissues and dividing cells are different (Bartholomae et al. 2011), and such differences depend on gene expression profiles in the target cells (Biasco et al. 2011).

Adenovirus

Biology

Adenoviruses are nonenveloped (without an outer lipid bilayer), icosahedral, lytic DNA viruses composed of a nucleocapsid and a linear double-stranded genome (Fig. 14.11a). Adenoviruses are capable of infecting both dividing and nondividing cells. More than a hundred (sero) types of adenoviruses have been identified to date. They are grouped into seven subgroups or species (A-G) based on genome size, composition, hemagglutinating properties, and oncogenicity. The adenoviruses serotype 2 and 5 are the most extensively studied and the first to be used as vectors for gene therapy. The adenoviral genome is a linear, nonsegmented dsDNA, between 26 and 45 kb, composed of six early (E1a, E1b, E2a, E2b, E3, and E4) and five late (L1, L2, L3, L4, and L5) genes. The early genes encode proteins necessary for viral replication and prevention of cell death, while the late genes encode proteins for virus assembly, release, and cell death. The genome of adenoviruses is flanked by hairpin-like inverted terminal repeats (ITRs), functioning as self-priming structures that facilitate primase-independent DNA replication (Arrand and Roberts 1979; Shinagawa et al. 1980). A packaging signal sequence promotes viral genome packaging.

Adenovirus infection typically begins with the binding of the fiber knob on the surface of the viral capsid to the CAR and major histocompatibility complex (MHC) class I

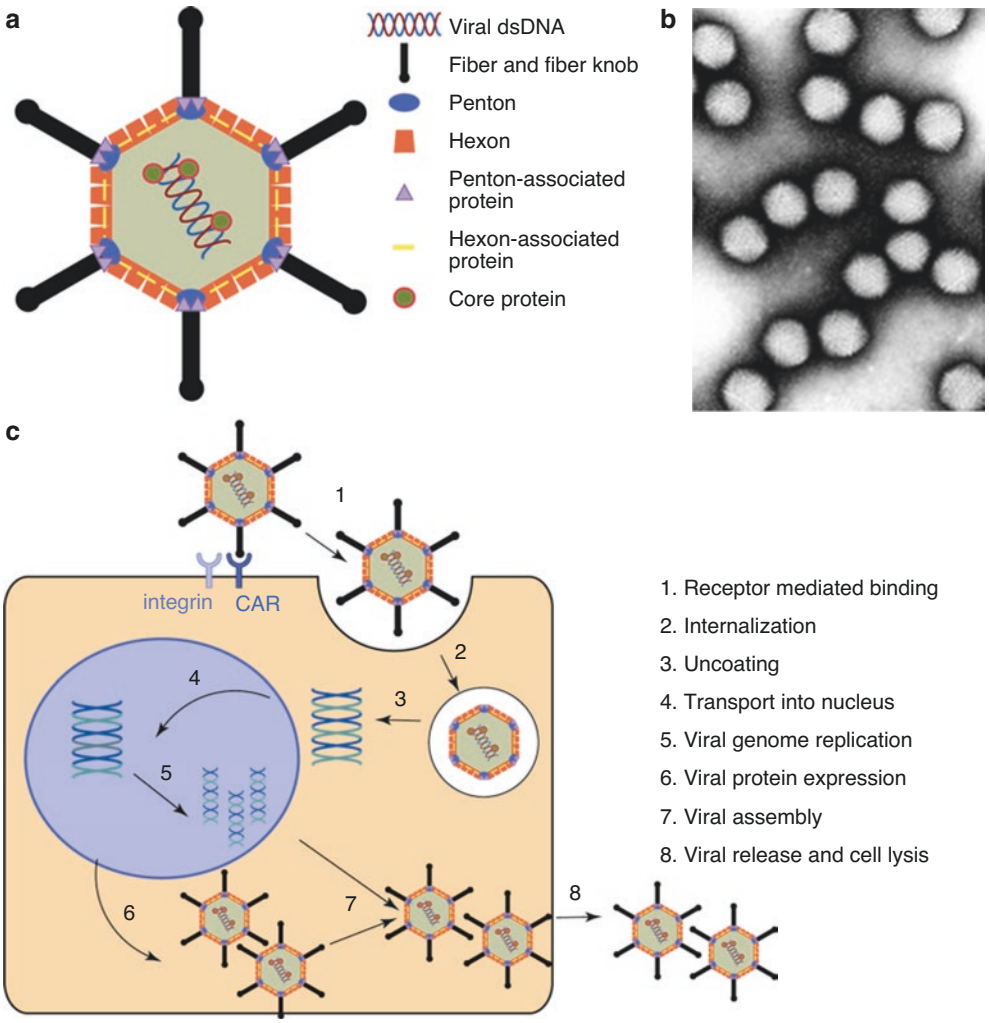

Fig. 14.23 The adenovirus. (**a**) Cross-section of an adenovirus particle. The virus consists of a double-stranded DNA genome encased in a protein capsid. The capsid is primarily made up of hexon proteins, forming 240 trimers. Penton proteins are positioned at each of the 12 vertices of the icosahedral capsid and serve as the base for each fiber protein. Hexon-associated and penton-associated proteins are the glue that holds these proteins together within and across the facets of the capsid. Core proteins bind to penton proteins and serve as a bridge between the virus core and the capsid. (**b**) Electron micrograph of intact adenovirus serotype 5 particles. (**c**) The adenovirus replication cycle. Adenovirus infection begins with the attachment of fiber proteins to cellular receptors such as coxsackie and adenovirus receptor (CAR), and integrins. Through receptor-mediated endocytosis, the virus enters the cytoplasm. In the endosome, capsid proteins are degraded, and viral DNA is released into the cytoplasm and transported to the nucleus for replication. After assembly into new viral particles in the cytoplasm, the host cell is lysed, and the viral progeny is released. In the case of gene therapy, recombinant replication-defective adenoviruses are used to transduce targeted cells. The genome is engineered to accommodate therapeutic transgenes transcribed to mRNA in the nucleus. Messenger RNA is then transported out of the nucleus and into the cytoplasm, where it is translated to therapeutic proteins

(Fig. 14.23). Several alternative entry receptors have been identified, including sialic acid, CD46, and Desmoglein-2 (Gaggar et al. 2003; Nilsson et al. 2011; Wang et al. 2011).

After initial binding, the penton base interacts with integrins on the cell surface to initiate a series of cell signaling processes allowing internalization via receptor-mediated endocytosis (Nemerow and Stewart 1999; Medina-Kauwe 2003). As a result, adenovirus particles enter the nucleus via the nuclear envelope pore complex as early as 30 min after initial cellular contact (Wiethoff and Nemerow 2015). Viral DNA replication and particle assembly in the nucleus starts 8 h after infection and culminates in the release of 10^4–10^5 mature virus particles per cell 30–40 h postinfection by cell lysis (Majhen and Ambriovic-Ristov 2006).

Adenoviruses as Gene Therapy vectors

To construct an adenoviral vector for gene therapy, the E1 and E3 regions of the viral genome are often removed. This both prevents viral replication and creates space to accommodate transgene cassettes. Adenoviruses have a large genome capable of accommodating large transgene cassettes. The adenoviral genome is also easily manipulated to

generate a vector with multiple deletions and inserts without affecting its transduction efficiency. Adenoviruses with both E1 and E3 inserts to simultaneously express two therapeutic genes have been reported (Panakanti and Mahato 2009). Moreover, adenoviruses with E1, E3, and E4 deletions and even "gutless" adenovirus (adenoviruses without viral coding regions) have been constructed to drive transgene expression (Armentano et al. 1995; Chen et al. 1997).

Other favorable characteristics of adenoviruses include that the biology of the virus is well understood, that recombinant virus can be generated with high titer and purity, that transgene expression from adenoviruses is rapid and robust, and that adenoviruses can infect a wide range of dividing and nondividing cells. Unfortunately, adenovirus genomes do not integrate into the host genome. While this minimizes the risk of insertional mutagenesis, gene expression is transient, making adenoviruses unsuitable for long-term correction of genetic defects.

The major drawback to the use of recombinant adenoviruses is the ability of the virus to elicit strong innate and adaptive immune responses and the existence of widespread preexisting neutralizing immunity in the population. Innate and adaptive immunity results in the killing of adenovirus-transduced cells and the production of antibodies to adenovirus, resulting in the clearance of the adenoviral vectors from the body (Dai et al. 1995). Preexisting neutralizing antibodies against one or more of the commonly used adenovirus (sero)types immunity in human populations, as a consequence of prior adenovirus infections, significantly reduce the efficacy of these vectors in both preclinical studies and clinical trials (Ertl 2005). To overcome this, researchers have started studying the use of human adenovirus types with low seroprevalence in the human population, such as Ad26. Importantly, these types seem to induce less potent immune responses than the most commonly studied Ad5 (Chen et al. 2010).

The immunogenicity of recombinant adenovirus raises serious safety concerns for its clinical applications. The massive immune responses caused by the administration of adenovirus could lead to multiple organ failures resulting in death. In 1999, a patient died 4 days after injection with an adenoviral vector. This was the first death of a participant in a clinical trial for gene therapy (Stolberg 1999). Another patient experienced a severe immune response syndrome characterized by multiple organ failure and sepsis and died soon after an adenoviral vector dose injection in 2003 (Raper et al. 2003). Preclinical studies also confirmed that the immune response generated by adenoviral vectors must be suppressed before a therapeutic effect can be expected. The transgene expression from adenovirus-transduced cells lasted for about 5–10 days, partially due to the clearance of the transduced cells by the host immune system (Lochmüller et al. 1996). Adenoviruses show an extended duration of expression when given to nude mice (mice with an "inhibited" immune system) or when an immunosuppressant is administered (Dai et al. 1995). Importantly, in some cases, the strong immunogenicity of adenovirus vectors benefits the therapy, e.g., in cancer or vaccines against pathogens. Less immunogenic adenovirus types such as Ad26 may not be the best choice in these cases. Alternatively, nonhuman primate-derived adenoviruses has been suggested as a source for vector development to avoid preexisting neutralizing immunity while maintaining a strong immune stimulation upon administration (Bots and Hoeben 2020).

A significant effort has been put forth to address the issue of the adenovirus-induced systemic immune response and potential regeneration of replication-competent adenovirus by engineering next-generation adenoviral vectors. First-generation adenoviral vectors were engineered by removing E1 and/or E3 to allow for transgene insertion of up to 4.5–6.5 kb. As the E1 region is vital for adenovirus replication, E1-expressing cell lines have been generated to produce these vectors, such as HEK293. E3 is dispensable for viral propagation in cultured cells.

Second-generation adenovirus vectors lack additional early gene regions (E2a/E2b/E4), enlarging the space for transgene cassettes to 10.5 kb. Like for first-generation adenoviral vectors, the gene deletions are compensated for by producer cell lines expressing the genes. However, titers of second-generation adenoviral vectors are typically lower than for first-generation vectors. Second-generation adenoviral vectors induce notably lower immunogenicity as less viral antigens are being produced, resulting in longer-lasting expression of the encoded transgenes. Nevertheless, the late genes that are still present in the adenovirus vector genome can still trigger undesired immune responses against the vector.

Third-generation adenoviral vectors are called "gutless," "helper-dependent," or "high-capacity" and carry none of the viral sequences except for the ITRs and the packaging signal. These vectors can accommodate ~36 kb of space for transgene cassettes. Production depends on additional helper viruses carrying loxP sites flanking the packaging signal and producer cells that express Cre recombinase. The viral proteins expressed by the genome of the helper viruses allow for replication and packaging. The Cre recombinase ensures the removal of the packaging signal from the helper virus genome to ensure that only the adenovirus genome can be packaged. Third-generation adenoviral vectors have even more reduced immunogenicity, longer-lasting transgene expression in the host cell, and a larger transgene capacity. The main challenge is to eliminate the helper virus from vector batches. Conditionally replicating adenoviral vectors carry tumor-specific gene promoters to make the viruses specifically replicate in tumors. Initially, these were generated by partial deletion of E1B, restricting genome replication to cells that lack p53 such as tumor cells. More recently, condi-

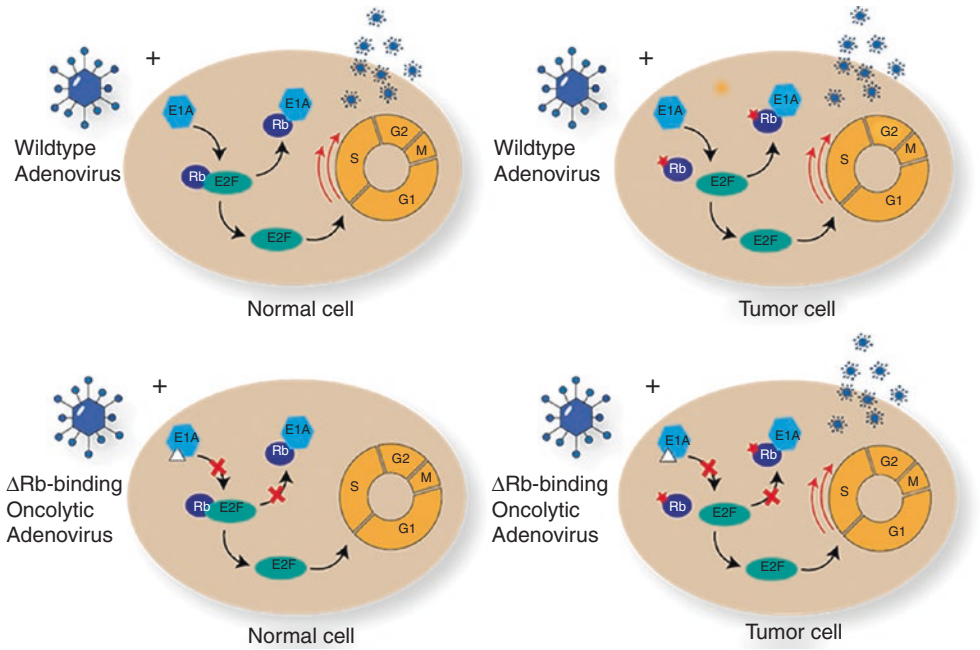

Fig. 14.24 Conditionally replicating adenoviruses. During infection with wild-type adenovirus, the viral E1A protein binds to Rb, releasing E2F. Consequently, the cell cycle progresses, facilitating viral genome replication and virus progeny production. Tumor cells often harbor aberrations in the Rb pathway, resulting in constitutively released E2F. This stimulates cell cycle progression into the S-phase independent of E1A binding to Rb, facilitating virus progeny generation. AdΔRb vectors harbor E1A proteins that are unable to bind to Rb. As a result, Rb remains bound to E2F in normal cells, preventing S-phase and viral genome replication. In tumor cells, however, E2F is already in a released state, so the cell cycle will progress, and virus progeny can be produced. As a consequence, AdΔRb vectors display tumor-selective replication. Courtesy of S.T.F. Bots

tionally replicating adenoviral vectors have been generated by removing a specific stretch of 24 amino acids from the E1A protein. These so-called AdΔ24 or AdΔRb vectors cannot bind to the retinoblastoma (Rb) protein. Rb normally retains E2F, preventing the cells from entering the S-phase and thereby replicating the genome. Cancer cells often have an aberrated Rb pathway and thereby facilitate the S-phase and AdΔRb replication independent from the Rb-binding activities of E1A (Fig. 14.24). These vectors have been widely studied in oncolytic virotherapy, which is more elaborately discussed later in this chapter.

Another hurdle for the use of adenovirus vectors in the human body is the generation of replication-competent adenovirus (RCA). Although the early genes responsible for viral replication and pathogenicity are already removed in the vector construction process, RCA can still be generated by homologous recombination if there is some overlap between sequences in the virus genome and the packaging cell genome. Although adenoviruses typically cause mild respiratory illness, RCA in clinical products could be life-threatening for immune-compromised patients. Several groups have observed the production of RCA from HEK293 cells caused by sequence overlap (Louis et al. 1997). Some new packaging cell lines with less overlap have been reported to overcome such problems. Moreover, there are strict rules for the purity of vector batches, e.g., the FDA recommends a maximum level of 1 RCA in 3×10^{10} viral particles. RCA assays have been developed to screen vector preparations for the presence of RCA.

Clinical Use of Adenoviral Vectors

Over 500 clinical trials have been initiated using adenovirus vectors (Bulcha et al. 2021). Approximately 50% of all gene therapy clinical trials involving viral vectors use recombinant adenoviruses, making them the most widely applied vector for gene transfer. China was the first to approve a gene therapy, Gendicine, in 2003, for the treatment of head and neck cancer (Pearson et al. 2004). In this adenoviral vector, the E1 gene is replaced by the tumor-suppressor p53. Since its approval, it has been studied in various additional cancers. In 2005, another adenovirus-based gene therapy, Oncorine, was approved in China for the treatment of nasopharyngeal cancer in combination with chemotherapy (Liang 2018). In this vector, E1B is partially deleted. Oncorine is currently also studied in other cancer types. However, both Gendicine and Oncorine have not been approved for clinical use outside of China. A recent addition to the list of marketed adenovirus-based gene therapies is nadofaragene firadenovec (Adstiladrin™). It is a nonreplicating AdV vector expressing recombinant human interferon alfa-2b that is administered

into the bladder to treat BCG-unresponsive nonmuscle-invasive bladder cancer. The results of a phase III clinical study showed that 55 out of 103 patients with bladder cancer (with or without a high-grade Ta or T1 tumor) had a complete response within 3 months of the first dose, and this response was maintained in 25 out of 55 patients at 12 months (Boorjian et al. 2021). The FDA approved it in December 2022. Additionally, the immunogenicity of adenovirus vectors has been used in vaccine development (Bulcha et al. 2021). In the last decade, human and chimpanzee Ad vectors have been developed and clinically tested for protection against Ebola, Influenza, or HIV. Especially the Ebola vaccines induced strong and specific cellular and humoral immunity that was long-lasting. Moreover, the global COVID-19 pandemic has prompted the development of additional adenovirus-based vaccines that deliver the SARS-CoV-2 spike protein. This led to the authorization of Vaxzevria (AstraZeneca, full market authorization) and Jcovden (Janssen, conditional market authorization) (see Chap. 15 Vaccines).

Adeno-Associated Virus (AAV)

Biology

The AAV genome is a 4.7 kb linear, single-stranded DNA molecule composed of two open reading frames (ORFs), rep, cap, and two inverted terminal repeats (ITRs) that define the start and end of the viral genome and packaging sequence. The rep genes encode proteins responsible for viral replication, while the cap genes encode structural capsid proteins. ITRs are required for genome replication, packaging, and integration.

The icosahedral AAV capsid is 25 nm in diameter. AAV is deficient in replication, and there are no packaging cells, which can express all the replication-related proteins of the AAV. Therefore, AAV requires coinfection with a helper virus, such as an adenovirus or a herpes simplex virus, to replicate (Fig. 14.25). Thirteen distinct AAV serotypes have been identified, and hundreds of AAV variants have been found in human and nonhuman tissues (Becker et al. 2022; Pupo et al. 2022). The biology of AAV serotype 2 (AAV2) has been the most extensively studied, and this serotype is most often used as a vector for gene transfer. Different serotypes are presumed to recognize different cell receptors and have distinct tissue and cell tropisms.

Suitability of Adeno-Associated Viruses for Gene Transfer

Recombinant AAV vectors have rapidly gained popularity for gene therapy applications within the last decades, due to their lack of pathogenicity and ability to establish long-term gene expression (Table 14.6). The viral genome is simple, making it easy to manipulate. In addition, the virus is resistant to physical and chemical challenges during purification and long-term storage (Croyle et al. 2001; Wright et al. 2003). The ability of the virus to integrate into the human chromosome was an initial concern, but eventually, it turned out that AAV only integrates into a fixed human genomic location called AAVS1, and the integration frequency of recombinant AAV is quite low (Surosky et al. 1997).

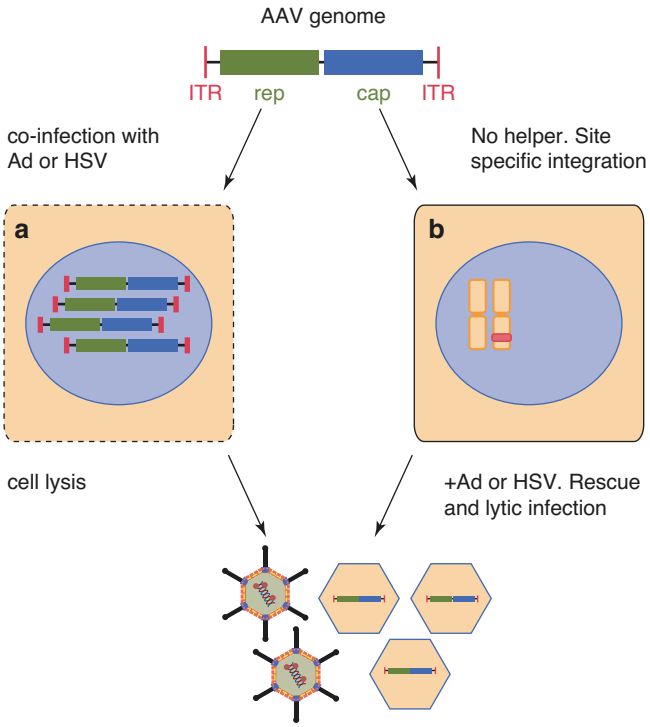

Fig. 14.25 Lifecycle of AAV. AAV can enter cells through receptor-mediated endocytosis. Dependent on pH, it escapes from the endosomes, then trafficking to the nucleus through the nuclear pore complex. Here, its genome is released from the capsid. Once in the nucleus, the virus can follow one of two distinct and interchangeable pathways. (**a**) In the presence of a helper virus (adenovirus or herpes simplex virus), AAV enters a lytic phase. The AAV genome undergoes DNA replication resulting in the amplification of the genome and production of progeny virions. The newly formed AAV viral particles and helper viruses are released from the cell by helper-induced lysis. (**b**) In the absence of a helper virus, it enters a latent phase. During this phase, part of the AAV vectors integrate into host genomic DNA at the preferred site AAVS1 while most AAV vectors persist in an extrachromosomal latent state without integrating into the host genome. The latent AAV genome cannot undergo replication and production of progeny virions in the absence of a helper virus. Similarly, the transgenes carried in the AAV genome are co-expressed without being copied by the host gene expression machinery. ITRs inverted terminal repeats, rep replication. AV Adenovirus; HSV, herpes simplex virus

The AAV vectors are produced by replacing the rep and cap genes with the transgene. Only one out of 100–1,000 viral particles is infectious. Apart from the production of AAV vectors being laborious, these vectors also have the drawback of limited packaging capacity (4.7 kb) for the transgene. Large genes are, therefore, not suitable for use in a standard AAV vector. To overcome the limited coding capacity, the ITRs of two AAV genomes can anneal to form a head-to-tail structure through trans-splicing between two

genomes, almost doubling the capacity of the vector (Yan et al. 2000).

Since recombinant AAV vectors do not contain any viral ORFs, they induce only limited immune responses in humans. Intravenous administration of AAV vectors in mice causes the transient production of pro-inflammatory cytokines and limited infiltration of neutrophils, in contrast to an innate response lasting 24 h or longer induced by aggressive viruses (Zaiss et al. 2002). However, despite the limited innate immunity elicited by AAV vectors, the humoral immunity elicited by AAV is still common. Depending on the serotype, it is estimated that up to 80 % of individuals are positive for AAV antibodies in the human population. The associated neutralizing activity limits the usefulness of AAV in certain applications. To overcome this, capsids have been isolated from nonhuman sources such as nonhuman primates or other vertebrate species. Alternatively, the vector can be retargeted by inserting or removing specific sequences from the AAV that are known to bind to certain receptors, or by directed evolution.

Clinical Use of Adeno-Associated Virus Vectors

The first clinical use of recombinant AAV was to transfer the cDNA of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) to the respiratory epithelium for treating cystic fibrosis (Flotte et al. 1996). Since then, hundreds of clinical trials employing recombinant AAV vectors have been initiated worldwide. The interest in AAV for gene therapy has been boosted by the approval of Luxturna and Zolgensma, two AAV-based gene therapies, for retinal dystrophy and spinal muscular atrophy, respectively. The first approval of AAV-based gene therapy was granted by the European Commission to Glybera® (alipogene tiparvovec), which encodes the gene for lipoprotein lipase deficiency for the treatment of patients with familial lipoprotein lipase deficiency (LPLD, synonym: type I hyperlipidemia) (Büning 2013; Salmon et al. 2014). However, this therapy was withdrawn in 2017 due to limited use. Numerous clinical trials using AAV gene therapy have demonstrated its safety and efficacy in neurological, musculoskeletal, hematological, ophthalmological, and metabolic diseases (Kuzmin et al. 2021). Efforts are being made to avoid vector accumulation in the liver and potentially related toxicity and to retarget the vectors to other organs than the liver, eye, nervous system, and muscles (e.g., the heart).

Nonviral Vectors

The inherent problems with recombinant viruses such as limited packaging capacity of transgenes, high production costs, and immunogenicity, a.o. reflected in the generation of neutralizing antibodies, have called for the design of efficient, nonbiological delivery methods for human gene therapy. These nonviral methods can be categorized into physical methods of gene transfection and physicochemical methods that make use of synthetic biological molecules (e.g., lipids, polymers, peptides, or sugars) to encapsulate, complex, or conjugate genetic material (further detailed below).

Like viral vectors, synthetic, nonviral vectors can be used to deliver genetic material for transient, episomal expression, stable transgene integration into the genome, and gene editing.

Transient expression involves the delivery of either plasmid DNA (pDNA) or mRNA. Longevity of expression is dependent on many factors, including the speed at which a cell divides, the stability of the mRNA or pDNA, and the type of nonviral vector that was used. In general, expression fades within 1-2 weeks but can be longer in slowly dividing cells.

Stable expression with synthetic vectors can be achieved by introducing pDNA encoding integration competent genetic elements. These can be of viral origin (Chiang et al. 2020), such as retroviral constructs or derived from engineered transposable elements such as Sleeping Beauty, piggyBac, and Tol2 (Sandoval-Villegas et al. 2021). In both cases, the integrase (for retroviruses) or transposase (for transposable elements) need to be delivered in trans with the DNA inserted, often provided in the form of mRNA. Stable integration has been described for these systems after electroporation or nonviral delivery, but the efficiency drops with the size of the DNA to be integrated.

Synthetic vectors are also very suitable for delivering the necessary components for gene editing. Compared to viral vectors, synthetic vectors offer several advantages for this. First, for some viral vectors, especially the adeno-associated virus, the packaging capacity is limited, which can be a limitation when large or more than 1 gene construct needs to be delivered. Synthetic vectors do not have this intrinsic limitation, as the size of these systems can be readily adjusted to accommodate bulky cargo. Second, unlike viral vectors, synthetic vectors can accommodate a mixed cargo of DNA, RNA, and protein. For example, lipid nanoparticles have been used to co-deliver spCas9 ribonucleoprotein complexes and single-stranded HDR templates to cells in culture, with high editing efficiencies (Walther et al. 2022). Viral vectors would rely on co-expression of the Cas9 nuclease and the sgRNA, each with their own promoter, making it quite bulky, and from a safety point of view, this is not desired. Thirdly, synthetic vectors do not suffer from vector-induced antibody responses, allowing redosing with the same synthetic vector. Despite these advantages, their clinical utility still suffers from low transfection efficiencies, which stems from the nonspecific uptake of the vector by epithelial barriers and extracellular matrix and poor delivery into the therapeutic target cells (Fig. 14.26). New emerging delivery systems and vector-constructing technologies try to address these issues.

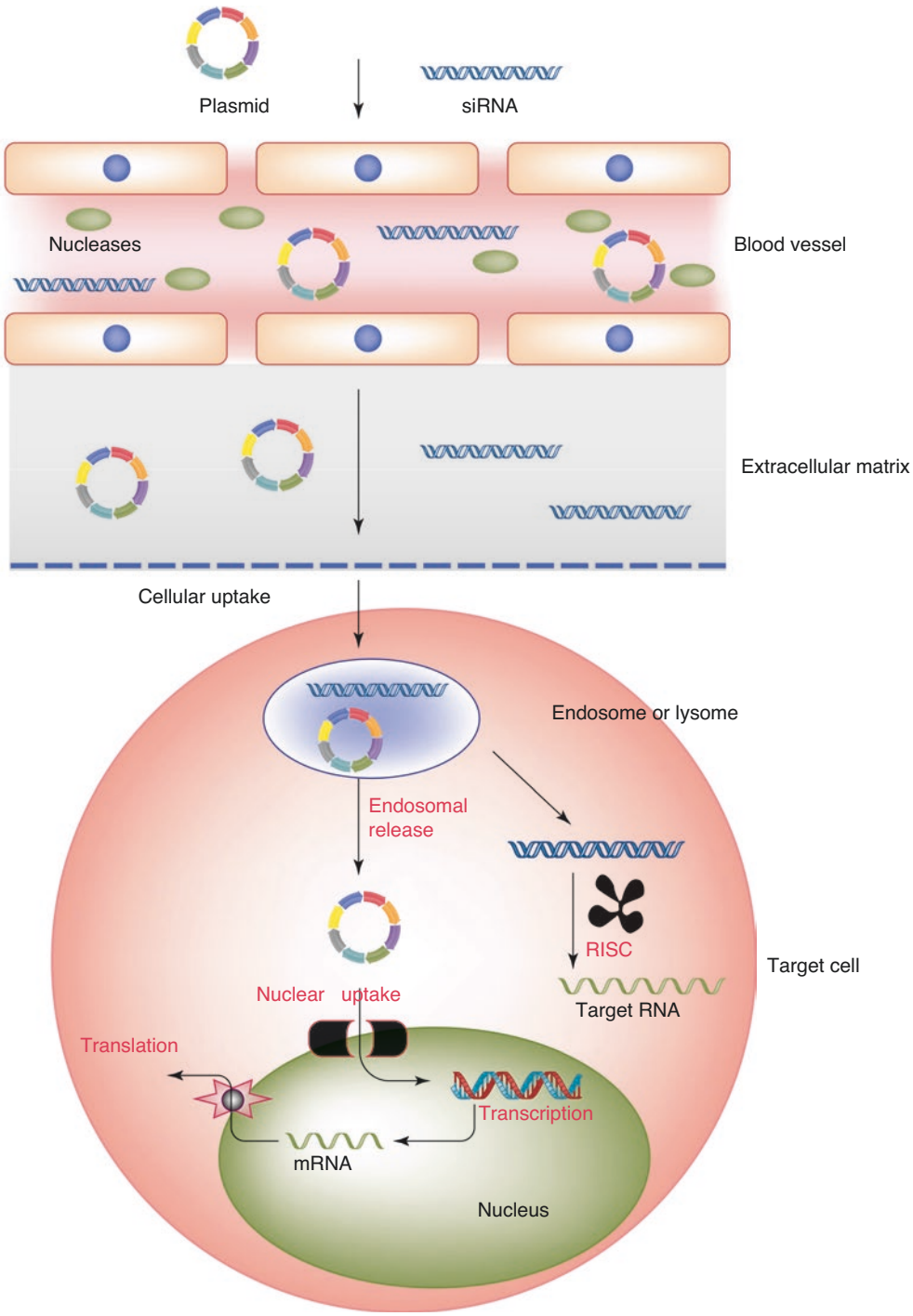

Fig. 14.26 Barriers to nonbiological gene delivery. Following systemic administration, the gene medicine (plasmid or siRNA) meets blood nucleases. Then they may traverse the blood vessel barrier and the extracellular matrix compartment before crossing the plasma membrane barrier. Upon entering the cell via receptor-mediated endocytosis, they are trapped in endosomes and need to be released into the cytosol. Endosomal escape is a major rate-limiting step in gene delivery. From Singh et al. (2011); with permission to reprint

Nonviral delivery methods can be subdivided into physical delivery methods and synthetic and biomimetic vectors for gene transfer.

Physical Methods for Gene Transfer

Physical methods involve the transfer of naked nucleic acids (DNA or RNA) by direct disruption of (target) cell membranes.

The earliest techniques to deliver recombinant DNA to cellular targets include microinjection, particle bombardment, and electroporation (Table 14.10). Microinjection, the direct injection of DNA or RNA into the cytoplasm or nucleus of a single cell, is the simplest and most effective method for the physical delivery of genetic material to cells. This transfects 100 % of the treated cells and minimizes waste of plasmid DNA. However, it requires highly special-

Table 14.10 Summary of nonbiological methods used for gene transfer

	Advantages	Disadvantages
Naked DNA	No special skills needed, easy to produce	Low transduction efficiency, transient gene expression
Physical methods		
Microinjection	Up to 100% transduction efficiency (nuclear injection)	Requires highly specialized skills for delivery
		Limited to ex vivo delivery
Gene gun	Easy to perform	Poor tissue penetration
	Effective immunization with low amount of DNA	
Electroporation	High transduction efficiency	Transient gene expression
		Toxicity, tissue damage
		Highly invasive
Sonoporation	Method well tolerated for other applications	Transient gene expression
		Toxicity not yet established
Laser irradiation	Can achieve 100% transduction efficiency	Special skills and expensive equipment necessary
Magnetofection	Safety of method established in the clinic	Poor efficiency with naked DNA
Chemical methods		
Liposomes	Easy to produce	Protein and tissue binding, transient gene expression
Micelles	Easy to produce and manipulate	Unstable
		Protein and tissue binding
Cationic polymers	High DNA loading	Transient gene expression, toxicity
	Easy to produce and manipulate	
Dendrimers	High DNA loading	Extremely toxic
	High transduction efficiency	
Solid lipid nanoparticles	Low toxicity	NA
	Controlled release and targeting	

NA not applicable

ized equipment and skills. Moreover, microinjection is unsuitable for in vivo or in vitro gene transfer into tissues or organs composed of many cells. Particle bombardment, or gene gun treatment, starts with coating tungsten or gold particles with plasmid DNA. The coated particles are loaded into a gene gun barrel, accelerated with gas pressure, and shot into targeted cells or tissues in a petri dish. Particle bombardment can be used to introduce a variety of DNA vaccines into desirable cells in vitro. However, particle bombardment has a low penetration capacity, making it unsuitable for in vivo gene delivery apart from easily accessible tissue, e.g., the skin. Electroporation generates temporary pores in the plasma membrane to transfer plasmid DNA to the cells by an externally applied high-voltage electrical field. Electroporation increases the gene transfer efficiency by 100–1000 folds compared to naked DNA solutions and has met with great success in laboratory practices and clinical trials (Wells 2004). For example, GMP-compliant electroporation devices have been developed to transfect cells in a closed flow system, thereby preventing potential contamination during transfection. Such closed systems are ideally suited for cell-based therapies in which cells need to be genetically modified prior to infusion into the patient (see "Cell Therapy" section) (Li et al. 2013). Electroporation can also be applied directly in vivo and has demonstrated safety and efficacy in clinical trials to treat melanoma, prostate cancer, and HIV infection (Daud et al. 2008; Vasan et al. 2011). Other physical methods for gene transfer include sonoporation, photoporation, laser irradiation, magnetofection, and hydroporation (Raes et al. 2021). However, because most of the physical methods induce stress and disruption of cellular structure and function, physical methods are less widely studied compared with the use of viral and synthetic vectors (see below) and are generally restricted to in vitro gene transfer of cultured cells or embryonic stem cells (Table 14.10).

Lipid-Based Vectors

Lipid-based vectors make use of cationic lipids to electrostatically bind negatively charged RNA/DNA to form complexes in the submicron scale (60-400 nm). Since the invention of lipofectamine in 1987, numerous cationic lipids have been synthesized and tested for gene delivery. Most of these cationic lipids are composed of three parts: (i) a hydro-

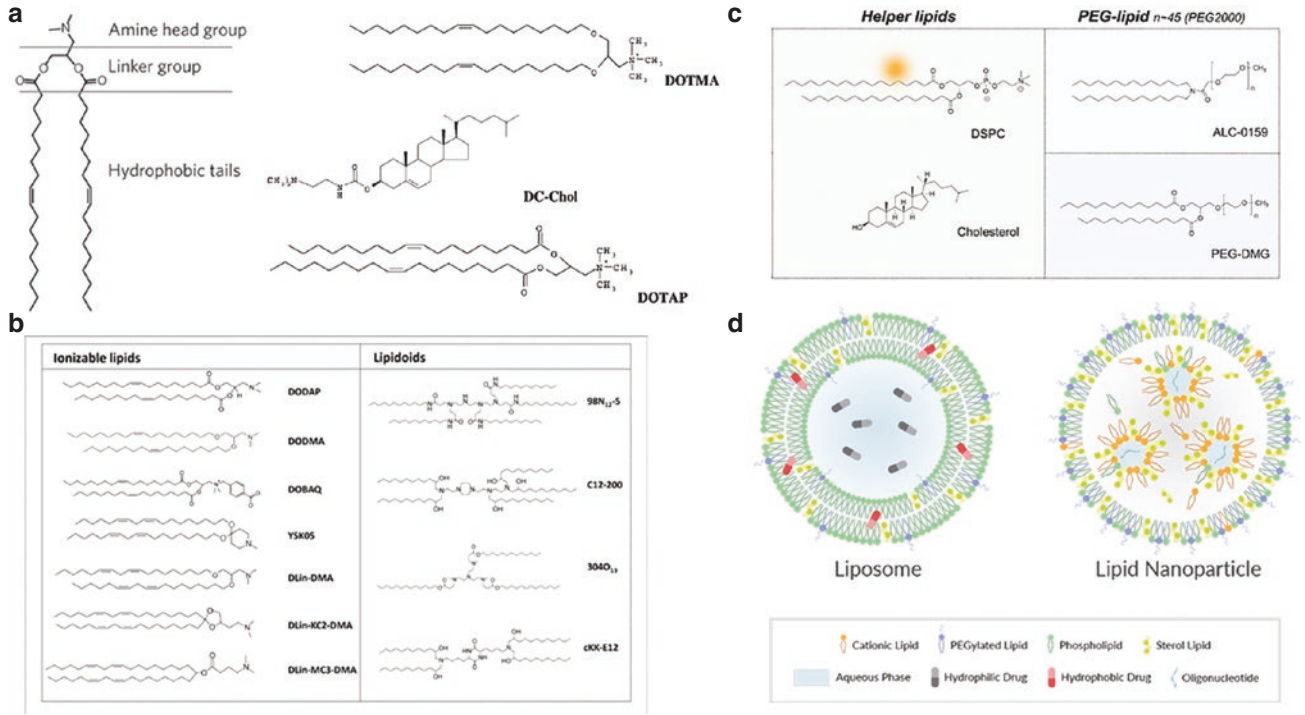

Fig. 14.27 Lipid-based vectors for gene delivery. The key component consists of permanently cationic (**a**) or ionizable lipids (**b**) mixed with neutral helper lipids (often phospholipids) and PEG lipids (**c**). Depending on the exact composition of the lipids and the ratio of nucleic acid cargo to lipid, these vectors can either form liposomes, having an aqueous interior, or LNPs with an interior mainly consisting of electrostatic complexes between RNA/DNA and (ionizable) cationic lipids (**d**)

phobic lipid anchor group; (ii) a linker group, such as an ester, amide, or carbamate; and (iii) a positively charged head group (Fig. 14.27) (Mahato et al. 1997). 2,3-dioleyloxy propyl-1-trimethyl ammonium bromide (DOTMA) and 3-β[N(NV,NV-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) are two commonly used cationic lipids with different structures. Cationic lipids are usually mixed with other lipids to change the structural properties as well as toxicity profiles of the formed complexes. Typically, these consist of cholesterol, neutral helper lipids, and lipids with a flexible poly(ethylene glycol) (PEG) polymer attached to their polar headgroup to increase nanoparticle stability and prevent rapid clearance once injected into the circulation.

Depending on the exact composition of the lipids and the ratio of lipids to nucleic acid cargo, the formed structures can either adopt a vesicular nature, containing lipid bilayers enclosing an aqueous core (liposomes), or form solid spherical structures in which the solid core consists of cationic lipid-complexed nucleic acids with some water, which are surrounded by a layer of neutral phospholipids and PEG lipids, the so-called lipid nanoparticles, LNPs.

Initially, permanently charged cationic lipids such as DOTMA were being used for in vivo transfection of pDNA, but direct exposure of cells to these highly cationic nanoparticles caused toxicity along with the activation of the innate immune system. This problem was partially solved by developing ionizable cationic lipids. In the LNP, the amino head groups are only charged at low pH (<6.5). In this way, nucleic acid encapsulation can be performed at low pH to enable optimal complexation, but once the LNPs are formed, and pH is neutralized, the LNPs hardly expose positive charges on their surface that could cause cytotoxicity. Screening of a vast library of such ionizable lipids and lipidoids (i.e., lipid-like materials) has led to the development of highly efficient LNPs for direct in vivo delivery of different therapeutic nucleic acids, including siRNA, mRNA, and pDNA (Sago et al. 2018) (cf. Chap. 5).

The mechanisms by which LNPs deliver their nucleic acid cargo is still a topic of investigation but involves endocytic uptake, followed by the exchange of lipids between the LNP and endosomal membranes, which in sporadic cases, leads to the endosomal escape of the nucleic acid cargo (Fig. 14.26).

To enhance endosomal escape and transport to and through the nuclear membrane, additional functional elements may be attached: for endosomal escape (pH-sensitive fusogenic peptides), for transport in the cytoplasm, and nuclear membrane passage (a nuclear translocation peptide).

LNPs are being explored for gene editing. Hereditary Transthyretin Amyloidosis is a rare genetic disorder that causes the amyloid formation of the TTR protein due to a point mutation in the *TTR* gene. By encapsulating an mRNA encoding spCas9 together with a sgRNA targeting the mouse transthyretin (*Ttr)* gene in the liver, a single intravenous administration of the gene could be effectively knocked out, leading to levels of TTR protein in the serum that were reduced by 97% and which persisted for at least 12 months (Finn et al. 2018). Interim results of a phase I study in patients suffering from hereditary transthyretin amyloidosis showed serum TTR level reduction up to 87%, after a single intravenous dose, with only mild grade 1 adverse effect (Gillmore et al. 2021).

Peptide-Based Vectors

Just like cationic lipids, cationic peptides condense DNA in a similar manner and can be used as gene delivery carriers. Poly(L-lysine) (PLL), a polydisperse, synthetic repeat of the amino acid lysine, was one of the first cationic peptides to deliver genes. However, an increase in the length of PLL leads to increasing cytotoxicity. Besides, PLL shows limited transfection efficiency and needs the addition of endosomolytic agents such as fusogenic peptides to facilitate plasmid release into the cytoplasm. Due to these issues, many researchers have turned to the development of PLL-containing "active" peptides and have met with some success (McKenzie et al. 1999). Such peptides offer many advantages over PLL, such as lower toxicity, precise control of synthesis, and homogeneity of peptide length.

Another class of peptides that have been extensively explored for gene delivery are the cell-penetrating peptides (CPPs). CPPs are short, synthetic peptides that facilitate cellular uptake and endosomal escape of molecules ranging from small chemical compounds, and nucleic acids to entire nanoparticles. CPPs can be categorized as those with a high abundance of cationic amino acids (lysine and arginine) or a sequence with alternating hydrophobic and polar amino acids, creating an amphipathic alpha-helical structure (Cf. Chap. 2). The latter are known for their endosomolytic activity. When complexed with nucleic acids, the nanocomplexes are taken up by endocytosis, where the peptides – because of the low pH – destabilize the endosomal membrane to release the cargo into the cytosol. The stability of the nucleic acid/peptide complexes can be greatly enhanced by the inclusion of a lipid tail to the C- or N-terminus of the CPP.

Despite efficient transfection of a variety of cells in vitro, peptide-based vectors are not yet suitable for systemic in vivo administration because of rapid destabilization, nonspecific plasma protein binding, and uptake by the reticuloendothelial system (Männistö et al. 2002). Another unique challenge for peptide-based gene delivery systems is cytosolic proteasomes, which degrade unneeded or damaged proteins by proteolysis. Co-administration of proteasome inhibitors is the most effective strategy to address this issue.

Polymeric Vectors

Synthetic and naturally occurring cationic polymers constitute another category of nonviral vectors. The advantage of synthetic polymers is that their architecture can be fully tailored: they can be made from biodegradable materials, the molecular architecture can be adapted from linear to branched to star-like dendrimers, and the density of cationic charges can be varied. Several polymers that have been studied for gene delivery are polyethyleneimine (PEI), poly[(2-dimethylamino) ethyl methacrylate (pDMAEMA), polyamidoamine (PAMAM) and biodegradable poly(β-amino ester) polymers (PBAE). Natural polymers, such as chitosan, dextran, gelatin, pullulan, and synthetic analogs, were also explored. Polymeric systems can generally be tailored to specific needs but require PEGylation to prevent undesired aggregation and rapid clearance in vivo. Some polymers, such as PEI, work via the postulated proton-sponge effect: the proton buffering capacity of such polymers at acidic pH causes the buildup of osmotic pressure when they reside in acidifying endocytic compartments, leading to rare endosomal burst events that enable the endosomal escape of the nucleic acid cargo. So far, only a few polymeric vectors for gene therapy have reached clinical stage development. A PEG–PEI–cholesterol lipopolymer is under clinical investigation for immunotherapy of ovarian and colorectal cancers through forced expression of the cytokine interleukin-12 (IL-12) (NCT01489371) (Thaker et al. 2015).

In another study, local delivery of a CRISPR-Cas9 ribonucleoprotein complex with a polymeric vector based on disulfide-crosslinked acrylates into the eye resulted in robust gene editing in the retina of nonhuman primates. It could potentially be used for local treatment of genetic eye diseases (Chen et al. 2019).

Extracellular Vesicles

Extracellular vesicles (EVs) encompass different types of lipid vesicles that are naturally secreted by cells, including exosomes, microvesicles, and apoptotic bodies, and may serve as a new type of delivery system for gene therapies (Yáñez-Mó et al. 2015; Varderidou-Minasian and Lorenowicz 2020) (Figure 14.28). EVs are secreted by almost every cell type and

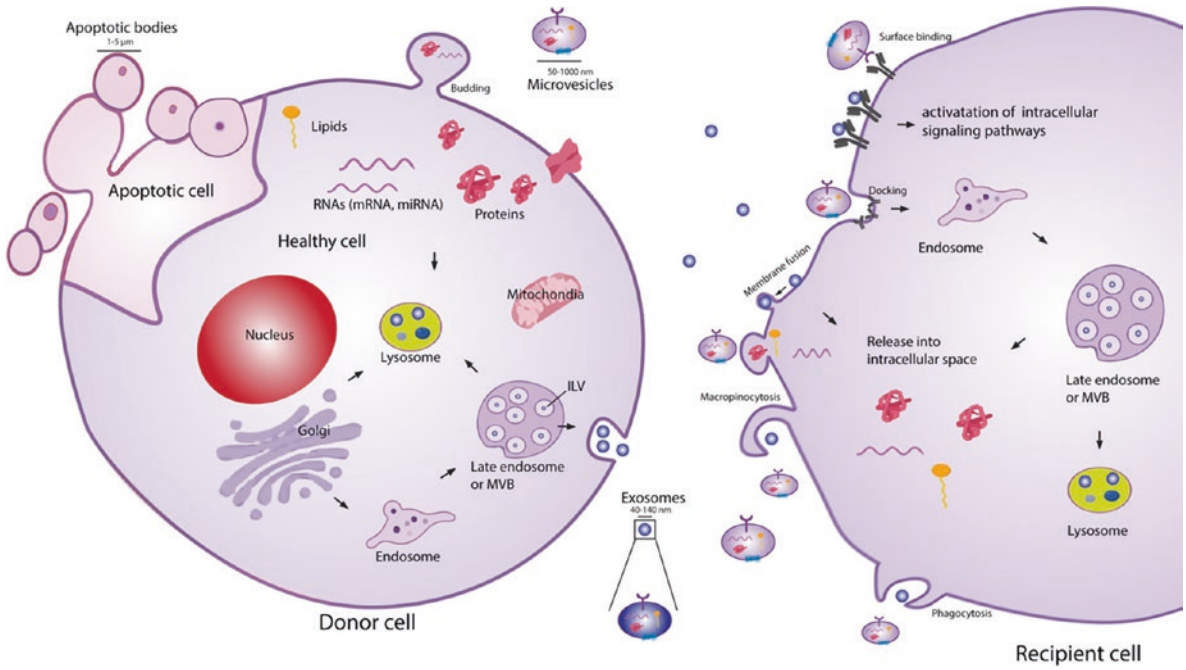

Fig. 14.28 Schematic representation of biogenesis, secretion, and uptake of major EV subpopulations: exosomes, microvesicles, and apoptotic bodies (Source: Varderidou-Minasian et al. 2020)

play pivotal roles in both physiology and pathophysiology. EVs naturally transfer biological payload (DNA, RNA, proteins, lipids) from cell to cell. Also, EVs can overcome cytotoxicity and immunogenicity issues associated with most viral vectors and synthetic nanoparticles, as demonstrated in preclinical models (Corradetti et al. 2021). These characteristics have boosted the development of EV therapeutics, which concerns the (ex vivo) production of EVs that are loaded with therapeutic drugs, proteins, small RNAs, mRNAs, or DNA (Liu and Su 2019). For example, Cas9-gRNA ribonucleoprotein can be loaded in EVs and functionally delivered in recipient cells, which, due to the large size of Cas9, is less feasible with AAV viral vector systems. The first engineered EV therapeutics have found their way into clinical trials, and initial findings on safety and efficacy are promising (e.g., to deliver cytokine profiles locally to tumor cells). Many developments are currently ongoing to fully explore the potential of EVs as a new type of delivery system for gene therapeutics. These not only focus on the engineering aspects to optimize the loading (and unloading) of payload but also involve targeting studies, specificity studies and development of scalable, clinical-grade EV production technologies.

Delivery Systems for CRISPR-Based Gene Editing

CRISPR-Cas9-based genome editing holds great potential to provide an entirely new class of therapeutics. However, to achieve effective therapeutic efficacy, the delivery of CRISPR-Cas9 components to the target cells of the patients is still a major hurdle and a prime topic for research. Several studies suggest an efficient delivery of the ~4 kb Cas9 from *Streptococcus pyogenes* into mammalian cells using adenoviral and lentiviral vectors (Eyquem et al. 2017). Nonviral approaches, including cationic polymer-based vectors (Platt et al. 2014), cationic lipid-based vectors (Zuris et al. 2015), and conjugated vectors (Ramakrishna et al. 2014), are also studied as delivery vehicles. For instance, CRISPR-Cas9 was used to target frequently mutated oncogene KRAS alleles in cancer cells and in vivo tumors using lentivirus or AAV expressing Cas9 and single guide RNA (sgRNA) (Kim et al. 2018). Also, more than 97% reduction in serum transthyretin level was achieved in mice when the CRISPR-Cas9 system was delivered using lipid nanoparticles (Finn et al. 2018). Other ex vivo delivery methods such as electroporation and nucleofection, are also extensively applied for the delivery of CRISPR-Cas9 components.

Disease Targets for Gene Therapy

In 2021 3000+ active gene therapy clinical trials were registered worldwide (Fig. 14.29) (John Wiley and Sons LTD 2022). Approximately 65 % of these trials are for cancer treatment. Treatment of monogenetic, cardiovascular, and infectious diseases each takes ~6-12 %, whereas treatment of neurological diseases is close to 2% (Fig. 14.29). Currently, gene therapy trials are primarily performed in the United States (58.3 % of all trials), China (10.1 %), the United Kingdom (7.3 %), and Germany (3.5 %). The geographical distribution of gene therapy clinical trials is summarized in

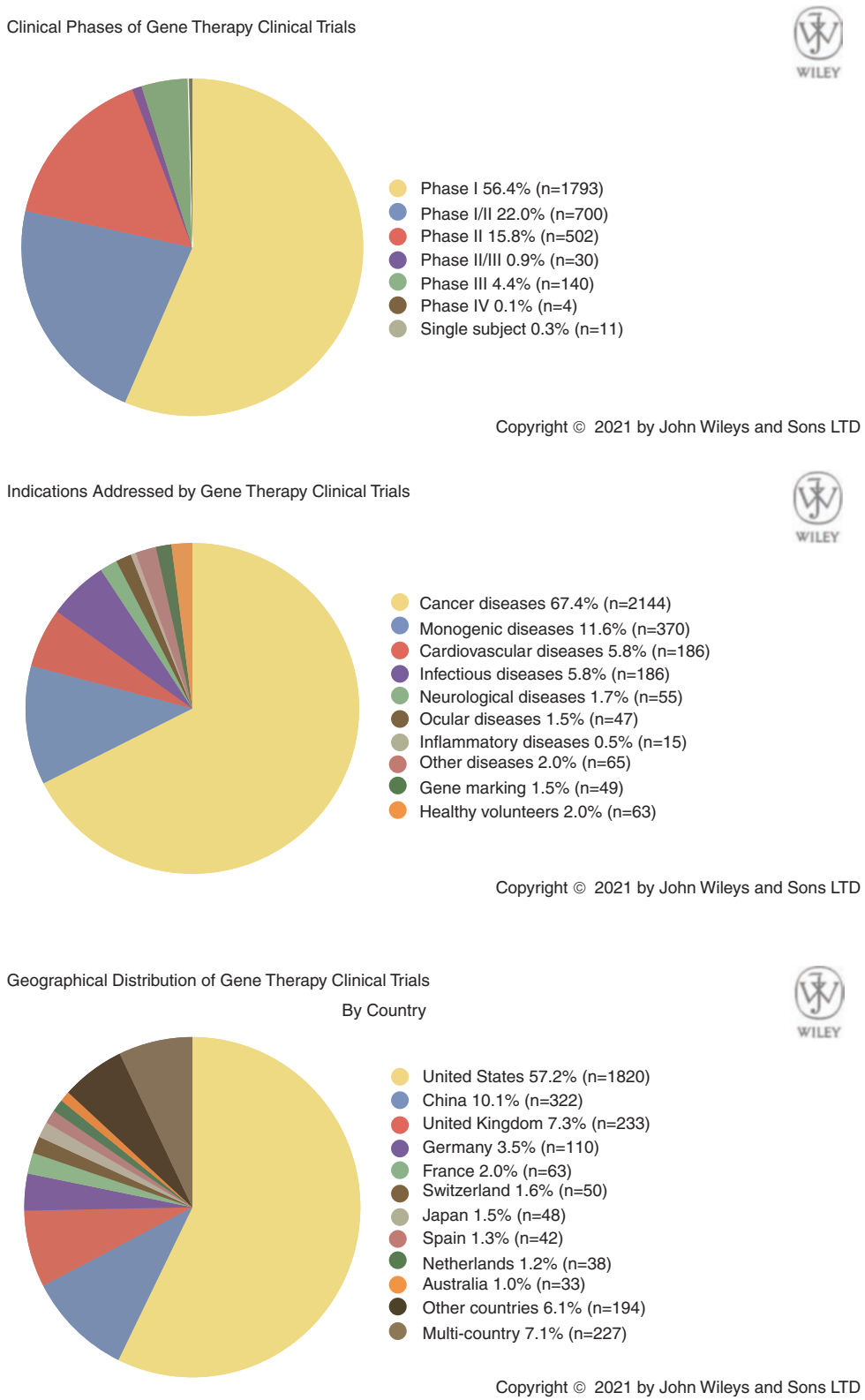

Fig. 14.29 Phases of gene therapy in clinical trials (John Wiley and Sons LTD 2022) Disease targets of gene therapy clinical trials (source: Wiley 2021). Other diseases include inflammatory bowel disease, rheumatoid arthritis, chronic renal disease, carpal tunnel syndrome, Alzheimer's disease, diabetic neuropathy, Parkinson's disease, erectile dysfunction, retinitis pigmentosa, and glaucoma International status of gene therapy clinical trials (source: Wiley 2021)

Table 14.11 Conditions for which human gene transfer trials have been approved

Cancer	**Other diseases**	**Cardiovascular disease**
Gynecological	Inflammatory bowel disease	Peripheral vascular disease
Breast, ovary, cervix	Rheumatoid arthritis	Intermittent claudication
Nervous system	Chronic renal disease	Critical limb ischemia
Glioblastoma, leptomeningeal carcinomatosis, glioma, astrocytoma, neuroblastoma	Fractures	Myocardial ischemia
Gastrointestinal	Erectile dysfunction	Coronary artery stenosis
Colon, colorectal, liver metastases, posthepatitis liver cancer, pancreas	Anemia of end-stage renal disease	Stable and unstable angina
Genitourinary	Parotid salivary hypofunction	Venous ulcers
Prostate, renal	Type I diabetes	Vascular complications of diabetes
Skin	Detrusor overactivity	Pulmonary hypertension
Melanoma	Graft-versus-host disease	Heart failure
Head and neck		
Nasopharyngeal carcinoma	**Monogenic disorders**	**Infectious disease**
Lung	Cystic fibrosis	HIV/AIDS
Adenocarcinoma, small cell, nonsmall cell	Severe combined immunodeficiency (SCID)	Tetanus
Mesothelioma	Alpha-1 antitrypsin deficiency	Epstein-Barr virus
Hematological	Hemophilia A and B	Cytomegalovirus infection
Leukemia, lymphoma, multiple myeloma	Hurler syndrome	Adenovirus infection
Sarcoma	Hunter syndrome	Japanese encephalitis
Germ cell	Huntington's chorea	Hepatitis C
	Duchenne muscular dystrophy	Hepatitis B
Neurological diseases	Becker muscular dystrophy	Influenza
Alzheimer's disease	Canavan disease	
Carpal tunnel syndrome	Chronic granulomatous disease (CGD)	
Cubital tunnel syndrome	Familial hypercholesterolemia	
Diabetic neuropathy	Gaucher disease	
Epilepsy	Fanconi's anemia	
Multiple sclerosis	Purine nucleoside phosphorylase deficiency	
Myasthenia gravis	Ornithine transcarbamylase deficiency	
Parkinson's disease	Leukocyte adherence deficiency	
Peripheral neuropathy	Gyrate atrophy	
	Fabry disease	
Ocular diseases	Familial amyotrophic lateral sclerosis	
Age-related macular degeneration	Junctional epidermolysis bullosa	
Diabetic macular edema	Wiskott-Aldrich syndrome	
Glaucoma	Lipoprotein lipase deficiency	
Retinitis pigmentosa	Late infantile neuronal ceroid lipofuscinosis	
Superficial corneal opacity	RPE65 mutation (retinal disease)	
	Mucopolysaccharidosis	

Information obtained from reference Ginn et al. (2018)

Fig. 14.29. General indications for gene therapy trials in the clinic are summarized in Table 14.11.

Cancer Gene Therapy

Most of today's gene therapy clinical trials are devoted to treating cancer. Gene therapy has the potential to target and destroy cancer cells in a way that is much more specific and tailored as compared to traditional cancer treatments with chemotherapy and radiation. This property has even become much more relevant in light of the current knowledge on the large intra- and inter-tumor heterogeneity of many cancer types, which advocates the use of therapeutics that attack cancer cells from multiple angles and in a directed fashion. The types of cancer gene therapies being explored vary widely and include oncolytic viruses, ex vivo immune cell modification (e.g., CAR and recombinant T cell receptor T cells), overexpression of pro-apoptotic genes (e.g., p53), introduction of suicide genes, suppression of oncogenes (e.g., using RNA interference), creating synergy toward other treatments (sensitization) and targeted interference with the cancer cell genome using CRISPR-Cas.

Correction of Genetic Abnormalities

In this approach, gene therapy corrects genetic abnormalities contributing to the malignant phenotype by replacing/reintroducing tumor-suppressor genes or downregulating certain oncogenic pathways. Understanding cancer at the molecular level is the starting point for gene correction in cancer therapy. The inactivation or activation of certain genes may contribute to tumor growth. Many cancer gene therapy clinical trials involve overexpression of tumor-suppressor genes such as p53, MDA-7, and ARF (Belete 2021). Mutations in the p53 gene are most commonly seen in a wide spectrum of tumors (Valente et al. 2018). Efficient delivery and expression of the wild-type p53 tumor-suppressor gene prevents the growth of human cancer cells in culture, causes regression of established human tumors in nude mice, or sensitizes existing tumors to the therapeutic effect of conventional chemotherapy and radiotherapy (Valente et al. 2018). The results from clinical trials indicated that the therapeutic effect of the adenoviral vector-based therapy Gendicine, the first gene therapy product, was promising in patients with head and neck squamous cancers (Xia et al. 2020). However, the results were only validated in China. Gendicine has also been used to treat various other cancers, which prolong overall survival when combined with other drugs. Although it does not show any adverse effects on the patients, vector-associated transient fever that lasts for only a few hours cannot be overcome in 50-60 % of the patients (Zhang et al. 2018). Efficient delivery of tumor-suppressor genes deep within tumor tissue is difficult, and restriction of gene expression in malignant tissue is challenging. Possibly this may be improved by the development of novel types of delivery systems. Gene silencing by this approach is also a limited success, especially when a prolonged effect is required. Tumor heterogeneity is another major bottleneck, and it will most likely require co-treatments or simultaneous "all-in-one-vector" delivery of multiple anti-cancer approaches at the same time. Despite these reservations, promising results have been obtained in clinical trials for different cancers, including prostate, lung, pancreatic, and brain tumors.

Immunotherapy

The past decade has witnessed some major breakthroughs in cancer treatment by applying immunotherapies. These consist of different approaches, all having in common that the patient's immune system is being modified or boosted to create additional antitumor responses. One example is the class of the oncolytic viruses (e.g., Imlygic®, talimogene laherparepvec, based on herpex simplex virus-1), which not only kill the tumor cells (in a specific way) but also induce a strong antitumor immune response, as outlined in more detail below. Other more recent developments are the autologous or allogeneic immune cell therapies, most strikingly CAR-T cells (covered in detail elsewhere in this chapter). This concerns the modification of immune cells outside the body (ex vivo) to direct them against tumor targets and subsequent infusion into the patient. Driven by the successes of CAR-T cells for the treatment of different hematological malignancies, many efforts are ongoing to improve the efficacy, e.g., to become active in so-called "cold tumors," that is, tumors with lower presence and influx of immune cells. Methods and technologies are improved to make this therapy more practical, easier, and cheaper. It is also being explored whether in vivo T cell therapy may be viable, which will require the use of vector systems that efficiently and safely deliver the transgenes into the immune cells inside the patient (Xin et al. 2022).

In recent years, a number of viral vectors have been developed that express therapeutic transgenes (Shaw and Suzuki 2019). Many of those represent oncolytic viruses (summarized below). Additionally, several finished and ongoing clinical trials have employed adenoviral vectors expressing tumor-associated antigens such as PSA or MAGEA3, or immunomodulatory molecules like IL-12 and interferon IFN-α/-β. As an example, nonreplicating adenoviral vectors encoding recombinant human interferon α-2b have been approved by the FDA for the treatment of BCG-unresponsive nonmuscle-invasive bladder cancer (Boorjian et al. 2021).

Oncolytic Viruses (Virotherapy)

Oncolytic virotherapy employs viruses that, either by nature or upon engineering, preferentially target tumor cells and induce antitumor immunity (Macedo et al. 2020). Moreover, it can be used to simultaneously deliver therapeutic genes into tumor cells. This represents a relatively novel anticancer approach that has gained interest in recent years. The first oncolytic virus that was approved was Rigvir, an ECHO-7 picornavirus. This virus was authorized in 2004 in Latvia for the treatment of melanoma patients. In 2005, China approved Oncorine, a genetically modified adenovirus (see "Adenovirus" section) for combination treatment with chemotherapy in nasopharyngeal carcinoma. More well-known is Imlygic (also called T-VEC), a modified herpes simplex virus, which was approved in the United States, the European Union, Australia, and Israel in 2015, for use in a subset of advanced melanoma patients (Kaufman et al. 2022). A number of other oncolytic viruses are currently under (pre)clinical investigation, including picorna-, adeno-, reo-, pox-, paramyxo-, rhabdo-, and retroviruses (Macedo et al. 2020). Their clinical antitumor effects as monotherapies, although long-lasting, seem moderate at best. Although combinations with other (chemo- and immuno-) therapies show promising results, alternative (nonviral) approaches with similar effects are often preferred. Many current research efforts are therefore focused on generating oncolytic viruses with enhanced potency. In doing so, viruses have, for example, been engineered with expanded tropisms, enhanced tumor selectivity, and/or increased immunostimulatory properties (Harrington et al. 2019). Additionally, efforts are made to evade circulating immunity in the human population by employing low-prevalent serotypes or nonhuman viruses (Uusi-Kerttula et al. 2015).

Monogenic Diseases

Great successes of gene therapy have been achieved in treating monogenic diseases (see Table 14.6 for a detailed list of approved gene therapy products). The approach has mainly been to apply gene augmentation or addition to restore a loss-of-function mutation, particularly successful for ex vivo applications. Severe combined immunodeficiency diseases (SCID) is a group of diseases in which ex vivo gene therapy has shown a lasting, clinically meaningful therapeutic benefit, e.g., gammaretroviral vector gene therapy for ADA-SCID (approved product Strimvelis™) and X-linked SCID. Gene-based therapy for other blood disorders, such as for β-thalassemia, has also been recently approved (betiglogene autotemcel/Zynteglo™). In inborn errors of metabolism, HSC gene therapy has been used to produce recombinant protein and shown beneficial effects in cerebral adrenoleukodystrophy (CALD, approved product elivaldogene autotemcel/SKYSONA™) and metachromatic leukodystrophy (MLD, approved product atidarsagene autotemce/Libmeldy™)(see Table 14.6) (Eichler et al. 2017; Fumagalli et al. 2022), and other metabolic disorders including Fabry, Gaucher, and mucopolysaccharidosis are underway.

In addition, in vivo gene therapy trials using AAV vectors for the treatment of monogenic diseases, such as spinal muscular atrophy (SMA), have been approved by regulatory agencies (onasemnogene abeparvovec – xioi/Zolgensma®). However, a rare but major risk of this type of in vivo AAV gene therapy is that it can cause acute serious liver injury or acute liver failure (Chand et al. 2021). It was also thought until recently that the safety profile of AAV vector integration is negligible, but recent studies indicate that long-term preclinical studies may be required to thoroughly understand these risks (Nguyen et al. 2021).

Other approaches have used CRISPR/Cas9 editing technology for blood disorders and inborn errors of metabolism. For instance, *BCL11A*, an erythroid-specific enhancer that represses γ-globin expression and fetal hemoglobin in erythroid cells, has been targeted in autologous CD34+ cells (Frangoul et al. 2021; Quintana-Bustamante et al. 2022). In another trial for transthyretin amyloidosis using lipid nanoparticles to deliver CRISPR/Cas9, misfolded transthyretin serum levels were lowered after a single dose and caused durable knockout (Gillmore et al. 2021).

Issues that have prevented gene transfer for monogenic diseases are (a) lack of suitable gene delivery technologies, (b) unfavorable interactions between the host and gene transfer vector, (c) complex biology and pathology of monogenetic diseases and target organs, and (d) lack of relevant measures, i.e., biomarkers, to assess the clinical efficacy and long-term efficacy of gene transfer. Challenges that remain in treating monogenic diseases are to induce gene expression sufficiently to correct or prevent further progression of clinical phenotypes without induction of host immune responses against the vector component or transgene product and minimize the risk of insertional mutagenesis for integrating vectors in target cells. Improvements in vector technology and advancements in the understanding of disease pathology will vastly improve methods for the correction of genetic diseases.

Cardiovascular Diseases

Cardiovascular diseases are the fourth largest group of diseases actively treated by gene therapy clinical trials (John Wiley and Sons LTD 2022). The current understanding of molecular mechanisms of cardiovascular diseases has uncovered many genes that could serve as potential targets for molecular therapies. For example, overexpression of genes involved in vasodilation such as endothelial nitric oxide synthase and heme oxygenase-1 or inhibition of molecules involved in vasoconstriction (angiotensin-converting enzyme, angiotensinogen) have reduced blood pressure in animal models of hypertension (Melo et al. 2005). Most clinical trials for cardiovascular diseases are designed for treating coronary and peripheral ischemia. Overexpression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) has been effective in myocardial and peripheral ischemia in preclinical studies (Shimamura et al. 2020). Despite the lack of significant benefit in several earlier clinical trials, VEGF gene therapy did show an excellent safety profile and improvement of symptoms in patients following adenovirus or plasmid intramyocardial administration in both pilot studies and long-term follow-ups (Stewart et al. 2006; Reilly et al. 2005). However, limited success was experienced in using gene therapy to treat cardiovascular diseases compared to other areas. The efficacy of gene therapy for cardiovascular disease will most likely be enhanced by strategies that incorporate multiple gene targets with cell-based approaches. Few of the gene therapy approaches for cardiovascular diseases are in phase II or III clinical trials. In 2019, a phase III clinical trial investigating the effect of intramuscular injection of pDNA encoding hepatocyte growth factor for the treatment of critical limb ischemia with ulcerations was successfully finalized, which led to the conditional approval of Collategene in Japan (Ylä-Herttuala 2019).

Infectious Diseases

Genetic vaccines based on DNA or mRNA, which are being discussed in Chap. 15 have shown to be very effective in the prevention of a number of infectious diseases, including COVID-19. For treating chronic infections, gene therapy approaches are being developed as well. Gene therapy for acquired immunodeficiency syndrome (AIDS) is the main application in this category. These interventions share the goal of inducing remission from HIV pathogenesis without the use of antiretroviral therapy (ART). The interest in gene therapy for an HIV cure was inspired by the elimination of the intact virus in Timothy Brown (also known as the Berlin patient) and Adam Casteljo (also known as the London

patient), who both received stem-cell transplants from a CCR5-negative donor to treat their underlying malignancies (Gupta et al. 2019). This has spurred the research on genome editing approaches of CCR5 and co-receptor CXCR4 using CRISPR-Cas to confer resistance to CCR5-tropic HIV strains. Other strategies focus on boosting the immune system to reduce or eliminate the HIV pool. Many gene therapy trials for AIDS involve ex vivo transfer of genetic material to autologous T cells using self-inactivating or conditionally replicating viral vectors to improve the immune system of the patients (Manilla et al. 2005; Levine et al. 2006). Other trials employed overexpression of HIV inhibitors such as RevM10 to increase $CD4^+$ T cell survival in HIV-infected individuals (Ranga et al. 1998; Morgan et al. 2005).

Besides HIV, gene therapy approaches are being developed against chronic infections with hepatitis B and hepatitis C virus, herpes simplex virus, malaria, and bacterial infections.

Neurological Diseases

Progress has been made in gene therapy for neurological diseases. For example, the approval of onasemnogene abeporvovecxioi (Zolgensma®) for spinal muscular atrophy (SMA), which uses an AAV9 serotype with a human survival motor neuron 1 (SMN1) gene, is infused into the circulation but crosses the blood-brain barrier to transduce affected neurons.

Other more commonly investigated neurological diseases for gene therapy are Alzheimer's disease (AD) and Parkinson's disease. For Parkinson's disease, both gene augmentation to restore loss of dopaminergic neurons and restoration of neurotrophic factors have been investigated in clinical trials using AAV vectors and lentiviral vectors (Serva et al. 2022).

AD can be divided into familial and sporadic, with the familial form having mutations in three major genes, amyloid precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2). Many genetic or environmental factors play a role in sporadic AD, which makes it difficult to apply therapies designed for monogenic disorders. Gene therapy trials have been performed using neurotrophic factors, such as nerve growth factor, to promote neuronal and synaptic repair, but these have been providing mixed results (Lennon et al. 2021).

In the neurogenerative disorder Huntington's disease, many clinical gene therapy trials have been performed aiming at lowering the protein Huntingtin (Htt). Abnormal conformation of Htt results in the toxic gain-of-function. There are approaches using antisense oligonucleotides, but this can only transiently downregulate Htt. AAV5 vectors have been used to deliver miRNA to stably reduce Htt expression long-term (Byun et al. 2022).

Another group of neurological diseases encompasses leukodystrophies, which are a heterogeneous group of genetic disorders affecting the white matter of the central nervous system. Both AAV gene therapies as well as HSC gene therapy approaches have been tested, targeting different cell types in the brain and showing promising results in preclinical and clinical studies (von Jonquieres et al. 2021).

However, delivery of gene therapy technologies to the CNS requires careful selection of vector type and route of administration for optimal biodistribution to the affected cell types requiring correction or modulation of gene expression.

Nonclinical Animal Testing Considerations

A full pre-/nonclinical testing program during drug development, as presented in Chap. 8 for mAbs, may not always be feasible or necessary for advanced therapies due to the nature of these products that consist of a heterogeneous population of human cells or tissues (see also Table 14.2). Generally, the pre-/nonclinical testing package entails studies to provide data on the following:

(i) safety (toxicity, including immunogenicity);
(ii) tolerance (local, systemic);
(iii) biodistribution;
(iv) persistence (duration of exposure);
(v) in vivo proliferation, maturation, and/or differentiation into an unwanted lineage of stem cells (ESCs, iPSCs);
(vi) tumorigenicity;
(vii) reproducibility;
(viii) biological activity (potency) in vivo and/or in vitro; in vivo mechanism of action
(ix) in vitro and in vivo efficacy studies to understand
(x) which cells/cell-subpopulations and cell characteristics have therapeutic value;
(xi) PK/PD to serve dose definition, e.g., number of (viable) cells;
(xii) PK/PD to serve route of administration and schedule;
(xiii) study duration to monitor for toxicity, and;
(xiv) safety of the surgical procedure for local delivery of cells/tissues.

Nonclinical animal safety (toxicology) and efficacy (pharmacology) studies pose significant challenges when applied to advanced therapies, e.g., for the following reasons:

1. Molecular incompatibility and immune rejection in xenogeneic human-animal combinations i.e., human tissues/cells tested in animal models. This is also true for genetically transduced cells, where the genetic modification leads to the expression of human protein(s), e.g., CAR-T cells.
2. Cellular immunotherapy to treat cancer (e.g., TILs) relies on the interaction of the cellular product with the patient's immune system for its effect. The in vivo immunological effect will very likely be different between species.
3. Cells do not undergo ADME in a way conventional medicinal products often do.

Table 14.12 Examples of animal and other pre-clinical models applied for assessment of safety, efficacy, and product potency testing

Animal and other model options	Example	Comment
Immunodeficient or immunosuppressed animal	NOD.SCID-rd1 mouse model of retinitis pigmentosa	See Chap. 9 for details on transgenic animal models
Animal disease model	Diabetic mouse model	Not always possible especially in case of immune based disease
Homologous animal model	AMD mouse model	Copy of human condition regarding pathology, symptoms and prognosis of disease. Use species specific autologous or allogeneic cells instead of human cells and apply the same manufacturing process to produce the animal cell based product; characterize the product to the extent possible; mimic the clinical setting in terms of route of administration, surgical procedure, and dose regime, to the extent possible
Homologous animal model plus use of a vector	ADA-SCID mouse model	See above plus vector encoding the animal homologue for animal cell transduction
Non-invasive whole animal modeling system	Magnetic resonance imaging (MRI) or computed tomography imaging (CTI) techniques	Cell fate/biodistribution studies in animals
Large animal model	Delivery of cells in the sub-retinal space of a pig's eye to train surgeons to safely administer cells in the eye of AMD patients; delivery of stem cells for treating spinal cord injury in pig model	Development of complex surgical procedures which would be technically difficult or impossible in small species
In-vitro assay system	Cell culture system to mimic cell migration upon immune stimulus	Potency test to characterize an advanced therapy

Without nonclinical safety and pharmacology data, it may be difficult to predict the potential safety and efficacy of the cell therapy product in a first-in-human clinical study. Therefore, alternatives should be investigated that could yield evidence of safety and, evidence of efficacy or at least paucity of efficacy, including the use of models explained in Table 14.12.

Relevant Animal Models

Mice are often the species of choice to study advanced therapies. They are relatively inexpensive, reproduce quickly, and can be easily manipulated genetically. However, the ability of mouse experiments to predict the effectiveness of advanced therapies remains controversial. The failure of many mouse models to mimic particular human diseases has compelled investigators to examine animal species that may be more predictive of humans. Larger animals, such as rabbits, dogs, pigs, goats, sheep, and nonhuman primates, are potentially better models than mice. They have a longer life span, which facilitates long-term (e.g., years) studies that are critical for some advanced therapy products with a lifelong effect. In addition, many physiological parameters, e.g., immune system properties that play an important role in the reaction of the host animal to advanced therapies, are much closer to humans than rodents. Large animals also have significant advantages regarding the number and types of cells or amounts of tissues that can be reproducibly isolated from a single donor animal and ex vivo manipulated in sufficient quantity for analysis and for various nonclinical applications.

In case animal safety data do not provide meaningful information based on which an extrapolation can be made to potential risks posed to humans, those studies may be (partially) waived by regulatory authorities. Study set-up and duration for evaluation of the toxicity and/or biodistribution have to be determined on a case-by-case basis and depend on, e.g.,

- product half-life, which may vary between hours–days and months–years, the latter for cells that engraft in a specific niche in the human body;
- potential alterations of cells over time upon administration;
- dose regime of single or repeat dosing over a period of weeks–months–years;
- chance for migration of the cells in the body to unwanted sites upon administration, e.g., local administration of an adult stem cell in the subretinal space of the eye may be safer than the systemic administration of an ESC/iPSC-derived product;
- type and number of ex vivo cell manipulations performed during manufacture, i.e., in case cells are expanded for multiple passages close to the point where these cells senesce, and animal studies should be performed with cells beyond the cell passage used to manufacture the advanced therapy).

Generally, genotoxicity and specific safety pharmacology studies are not conducted for cell and tissue-based products unless there is a reason for concern, e.g., the use of a novel excipient or novel route of administration for an approved excipient. In addition, reproductive toxicity studies are only required when there is a potential risk for exposure to the reproductive organs. And finally, literature data may be used to support the (lack of) animal data. See Herberts et al. (2011) and Vestergaard et al. (2013) for further reading.

Clinical Testing Considerations

For investigating the safety and efficacy in humans, generally, the same principles apply to advanced therapies as to other medicinal products (see Table 14.4). However, considering the nature and complexity of the products and potential risks and benefits, there are some unique aspects to the clinical programs (Mount et al. 2015):

1. Different set-up of trials compared to most conventional medicinal products:
   (a) First-in-human trials are always in patients and never in healthy volunteers;
   (b) A seamless development path rather than the traditional route of separate formal phase I (safety), phase II (hint of efficacy), and phase III (safety confirmation and efficacy) studies.
2. Traditional PK (ADME)/PD studies may not be feasible.
   (a) Dose (defined as the number of cells/mL; the number of cells/kg body weight) escalation studies may not be feasible as there may not be clear dose-response correlations. A low-, medium-, and high-dose is often selected based on literature data concerning the number of cells that have historically been administered to humans.
   (b) Advanced therapies are frequently administered intravenously and rapidly cleared via the lungs, spleen, and liver (Leibacher and Henschler 2016). Other possible routes are intranodal (DCs to treat rheumatoid arthritis) or local administration via a surgical procedure, e.g., into the eye, brain, spinal cord, or knee.
3. For safety evaluation, the following risks may need to be taken into account, depending on many factors, including the type of product, cell differentiation status upon administration, cell proliferation capacity, cell source being autologous, allogeneic or xenogeneic, the half-life of the cells in the body/lifelong persistence, site and method of administration/implantation, quality of the starting material (derived from a healthy donor or very sick patient), and disease environment(s) which cells may encounter in the patient's body:
   (a) Tumor formation (tumorigenicity), e.g., in case of ESC- and iPSC-derived products which are ex vivo expanded and differentiated;
   (b) Potential adverse reactions at the site of administration, e.g., dimethyl sulfoxide (DMSO) related side effects upon i.v. administration;
   (c) Cells, being subvisible particles, make it difficult to assess subvisible particles potentially present in the product. These foreign particles may damage the tissue upon administration, e.g., in the subretinal space of the eye;
   (d) Inflammatory responses and infections (e.g., side effect of CAR-T cells);
   (e) Implantation procedure for cells or 3-D tissue replacement therapies using a complex surgical procedure, e.g., to administer cells in the subretinal space of the eye, in the spinal cord, or in the brain; 3-D cultured trachea placed in the throat;
   (f) Immuno-mediated side effects (CAR-T cells may cause cytokine release syndrome);
   (g) Immunogenicity, which may depend on:
   – Relative immune privilege of the administration site (e.g., eye);
   – Allelic differences between product and patient cells (e.g., allogeneic dendritic cells);
   – Immune competence of the patient;
   – Need for repeat dosing (more doses may increase the chance of immune rejection of the advanced therapy);
   – Maturation status of the cells (e.g., ESCs).

   Advanced therapies derived from an allogeneic cell source often require immune-suppressant medicines to be administered together with the cell-/tissue-based product. However, some allogeneic cell-/tissue-based products, such as MSCs, have shown relatively low immunogenicity profiles, in part due to the short half-life of the cells in the body. See more details below.
4. Selecting the right patient population for the initial clinical program is challenging as there is a tension between choosing the patients most likely to benefit from an efficacious advanced therapy (e.g., early-stage cancer patients) and limiting the risk to which patients are exposed to the unlicensed therapy (late-stage cancer patients who may not benefit from the therapy at all due to their severe illness).
5. Establishment of surrogate biomarkers for efficacy assessment may be needed to predict long-term clinical outcomes of cells that may persist in the body for years e.g., CAR-T cells which engraft in the peripheral blood and bone marrow and transduced CD34+ cells, which engraft in the bone marrow.

Table 14.13 Example of clinical trials with pluripotent stem cells (hESCs and iPSC), adapted from Trounson and McDonald (2015) and Ilic et al. (2015)

Indication	Active substance	Trial sponsor (country)
AMD	hESC-derived RPEs	Chabiotech (South Korea)
DryAMD; myopic AMO; Stargardt's macular dystrophy	hESC-derived RPEs	Ocata therapeutics (USA)
WetAMD	hESC-derived RPEs	Pfizer (UK)
DryAMD	hESC-derived RPEs	Cell cure neurosciences (Israel)
Type I DM	hESC-derived pancreatic endoderm cell	Viacyte/Johnson& Johnson
Heart failure	hESC-derived CD^{15+} $lsl\text{-}^{1+}$ progenitors	Assistance Publique-Hopitaux de Paris (France)
Parkinson's disease	Human parthenogenic-derived neural stem cells	International stem cell Corp. (Australia)
Spinal cord injury	hESC-derived oligodendrocyte precursors	Asterias Biotherapeuticcs (USA)
WetAMD	hESC-derived RPEs	The London project to cure blindness (UK)
WetAMD	iPSC-derived RPEs (autologous)	Aiken institute (Japan)

AMD age-related macular degeneration, *RPEs* retinal pigmented epithelial cells

6. Particularly for genetically modified cells, which may persist in the body for many years or lifelong, long-term (10–20 years) patient follow-up for safety, efficacy, and durability monitoring may be necessary.

Immunological Considerations in Advanced Therapy

The potential application of adult stem cell-based medicinal products derived from allogeneic sources and hESC-based therapies is limited by risks for graft-host rejection issues, as with all therapeutic strategies based on cell, tissue, and organ transplantation, unless the transplant is derived from an autologous source. A way to overcome this challenge is the use of a device to protect the allogeneic cellular product from the host immune system. An example of this strategy is Viacyte's cell-based combination product, where the hESC-derived β-islet progenitors are contained in the Encaptra® cell delivery system, which is placed subcutaneously (see Table 14.13) and later Fig. 14.34). The additional advantage of this system is that cells cannot migrate in the body to unwanted sites, and the device can be taken out in case of, e.g., tumor formation. The disadvantages of such an immune-protective device are fibrosis and the lack of vascularization around the device, which is required for cell viability and insulin production. Certain human body sites have immune privilege, i.e., they tolerate the introduction of nonself-antigens without eliciting an inflammatory immune response. These sites include the eyes, the testicles, the fetus, and certain tumors. There is debate in the cell therapy world regarding the immunogenicity of allogeneic MSCs (Ankrum et al. 2014; Consentius et al. 2015). Clinical trials with standardized immune monitoring programs and a better understanding of the in vivo mode of action of allogeneic MSCs may provide answers.

Administration of drugs to suppress the immune response is standard practice for patients undergoing transplantation, but with immunosuppression come side effects. The hope is that iPSC technology (see above) may overcome rejection problems for which several products are being tested in the clinic (Kim et al. 2022). Another approach is to bank a collection of ESC lines that allows the selection of a matched ABO and HLA haplotype or a close match (Lui et al. 2009). It has been estimated that with a bank of 70–100 ESC lines, a partially matched ESC line that is adequate for each recipient can be chosen. The downside of this approach is that at the time the cell lines are banked, it may not be clear yet for which diseases they will be used in the future, hence what the critical parameters are to characterize the banks, for example, purity of the cells, stability, potency, viral safety, see (Bravery 2015). Preparing cell banks, extensive testing and long-term storage under frozen conditions are very expensive undertakings.

Manufacturing and Testing Considerations

Manufacturing

Cell and tissue-based products are distinct from traditional biopharmaceuticals in that the modified cell/tissue itself is the active ingredient in the medicinal product rather than "simply" the means by which the cells produce an active ingredient (e.g., a recombinant protein; a viral vector). However, many of the production platforms, cell culture media, storage and transport bags, and product excipients and primary containers have been established for traditional

cell-based recombinant protein manufacturing processes (cf. Chap. 4) and can be readily applied to these innovative products.

Since the vast majority of advanced therapies contain viable cells/tissue that can be easily destroyed through sterilization procedures and cannot be sterile filtered (≤0.2 μm filter pore size), as cells have a size of 10–30 μm on average and tissues are even bigger, the manufacturing of these products must take place under aseptic conditions. For nonsterile raw and starting materials and excipients, additional steps may need to be taken to ensure subsequent aseptic manufacturing, e.g., heat inactivation, gamma-irradiation or sterile filtration of the material. The facilities, equipment, raw materials, viral vectors, and cells/tissues used must be of suitable quality to allow for good manufacturing practice (GMP) production of the drug product for human application. At every stage of production, materials and the final product should be protected from microbial, viral, and other contamination. The manufacturing of advanced therapies typically requires many or all of the following "manipulation" steps, see Table 14.14.

Control of the Manufacturing Process

As with manufacturing process of biologics, process variables need to be chosen carefully and monitored to allow for adjustments to the process and to ensure a product of high quality is consistently produced. Process variables assessed are, e.g., medium perfusion or exchange rate, feeding regime, biomass, stirring speed, pH, dissolved oxygen (DO), and lactate production. Particularly in the case of open and manual culture steps, this is challenging because any handling of the cells/tissue may impact the quality of the viable material and could potentially contaminate the culture system. Examples of fully closed production systems enabling different manipulation steps in one system are the CliniMACs Prodigy® and the Octane Technology (see Figs. 14.30 and 14.31, respectively).

A fully closed processing system is the CliniMACS Prodigy. This single-use device performs all manufacturing steps (i.e., cell wash, enrichment, activation, genetic modification, expansion, final formulation, and sampling). This contrasts with other manufacturing approaches, which use separate machines for cell culture, cell washing, and other steps in the production chain.

Table 14.14 Typical advanced therapy manipulation steps and equipment used for each step

Manipulation step	Equipment used (examples)
Collection or generation of autologous or allogeneic donor cells; collection of tissue biopsy (i.e., starting material). This step is not considered a GMP manufacturing step and takes place outside the GMP facility at a clinical site	Bone marrow aspiration system, surgical procedure, apheresis/leukopheresis system (Fig. 14.35)
Isolation of specific cell population(s). This is usually where the GMP manufacturing process starts	Knife; fluorescence-activated cell sorting (FACS) (see below): positive/negative selection by e.g., magnetic-activated cell sorting (MACS®) technology (microbeads and column); Elutra®; LOVO spinning membrane filtration device
Cultivation, expansion, and/or (genetic) modification of cells; tissue culture	Cell culture systems (see Chap. 4)
Cell differentiation	Specific raw materials, such as growth factors, are added to the culture medium manually or automatically
Purification of desired cell population(s); purification of tissue	Counter-flow centrifugal elutriation (Ficoll).This technique separates cells by size and density while maintaining cell viability. Cell enrichment kit for the magnetic separation of the desired cells by negative selection. It utilizes antibody magnetic bead complexes. Undesired cells are bound by the antibody and then magnetic beads that, when placed in a magnetic field, leave the desired cells untouched and free in the medium. The same principles and systems can be applied as for isolation of specific cell population(s) (see above)
Cell harvest and cell wash/cell concentration; tissue harvest and wash	Centrifuge; fluidized bed + elutriation-closed system (K-Sep); tangential flow filtration (TFF) technology; spinning-membrane filtration;
Formulation of the harvested cells in excipient mixture; formulation of tissue	Manually; mixing station with disposable bag set-closed system (Invetech)
Filing in the primary container of cell suspension; transfer of tissue to primary container (this is considered the drug product (DP)	Manual vial filling, stopping, and capping (Flexicon pump); manual bag filling and sealing; (semi) automated vial filling (FPC50, Flexicon system)
Labelling of the primary container	Manually; automatically with labelling machine
Short/long term storage of the DP	Refrigerator; controlled rate freezer; freezer, cryopreservation tank
Shipment of the DP to the clinical site	Temperature controlled shipment in cool box, on dry ice, in cryogenic Dewar
Handlings of the DP at the clinical site to allow for administration of the DP to the patient (e.g., thawing, washing, mixing with other ingredient)	Plasmatherm controlled temperature rate dry thawing instrument; centrifuge, mostly manual handlings

The manufacturing process for advanced therapies parallels the processes for *E. coli*/mammalian production cells described in Chap. 4 for therapeutic proteins. But they differ considerably from those processes at a number of critical points. On top of that, the various types of cell therapy products vary widely from each other. Below are examples of manufacturing process flow charts for three different types of advanced therapy medicinal products:

Fig. 14.30 Miltenyi's CliniMACs Prodigy closed processing system for cells grown in suspension (DCs, T cells)

1. Off-the-shelf or nonoff-the-shelf MSC production process, as described below and presented in (Fig. 14.32);
2. Non off-the-shelf CAR-T production process, as this procedure is a prime example of "personalized medicine" (see Chap. 9) the complexity is caught both in the text below and shown in Fig. 14.33;
3. Off-the-shelf human ESC-derived prebeta cell production process, as described below and presented in Fig. 14.34.

Manufacturing of MSC Product

The manufacturing of an off-the-shelf (allogeneic) or nonoff-the-shelf (autologous or allogeneic) cell-based product, e.g., MSC-derived product, is a multi-step process with slight modifications for each specific product (see Fig. 14.32).

- Step 1: Starting material procurement via bone marrow (BM) aspiration (1a) or adipose tissue biopsy (1b) from a healthy donor (allogeneic cell source) or patient (autologous cell source). Other sources of MSCs are not discussed here. The donor (healthy person or patient) is tested for specific human viruses before donating the starting material.
- Step 2: Mononuclear cell separation from BM (2a) using separation techniques; adipose tissue digestion using enzymes, such as collagenase (2b).

Fig. 14.31 Octane Technology, a fully closed production system for scale-out of autologous or allogeneic tissue- and cell-based products

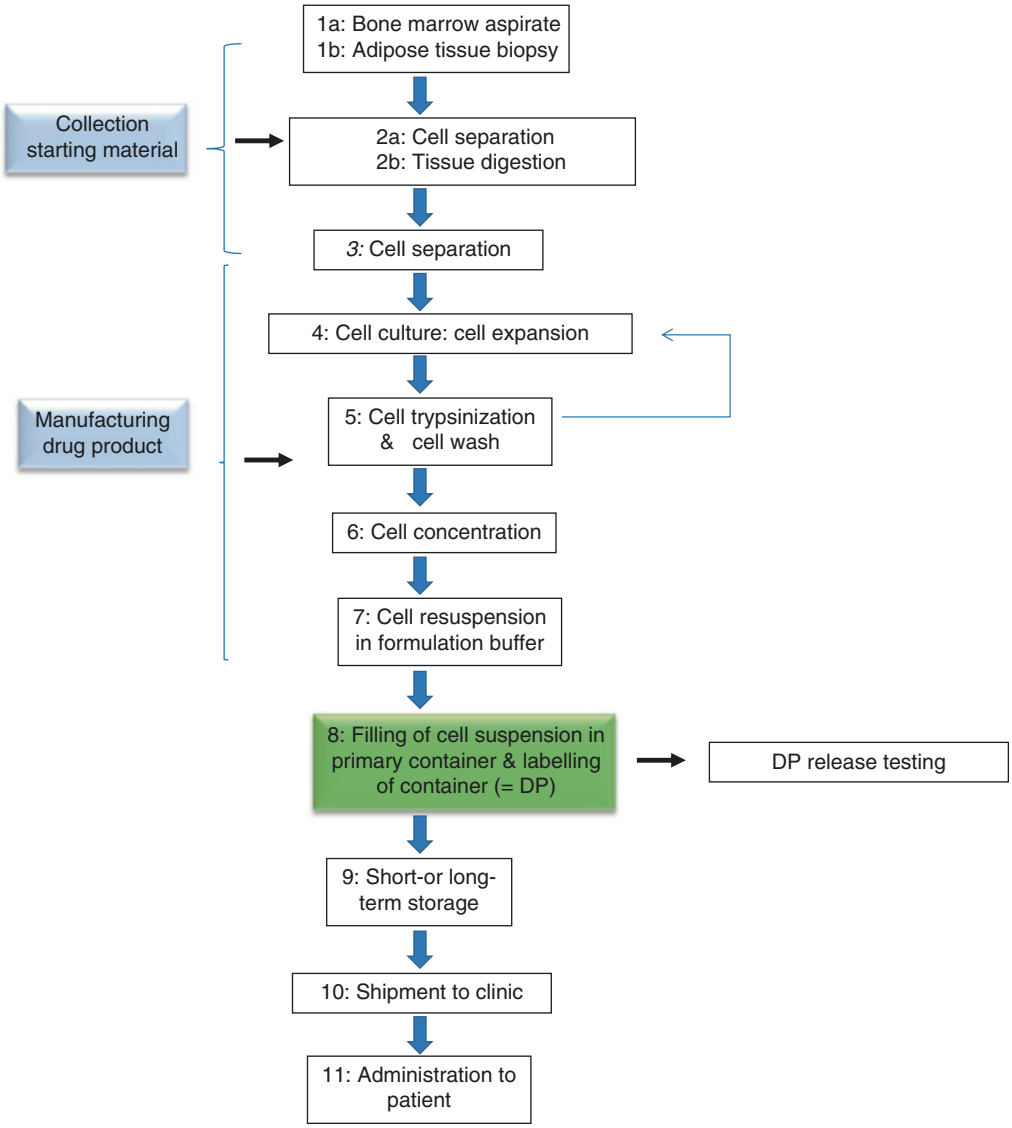

Fig. 14.32 Flow diagram of a manufacturing process for an off-the-shelf (allogeneic) or non-off-the-shelf (autologous or allogeneic) cell-based product based on adherent cells which do expand ex vivo, such as MSCs

- Step 3: Mononuclear cell separation from digested adipose tissue.
- Step 4: MSC expansion: MSCs are adherent cells and can, therefore, either be cultured in a culture flask (2D culture) or on micro-carriers in suspension culture (3D culture). Cells grow and multiply via mitosis and meiosis. By selecting the appropriate surface and culture medium, and culture conditions, unwanted cell populations do not adhere and are separated from the wanted cell populations.
- Step 5: Cell detachment from the surface via trypsinization. Cells are washed to remove dead cells, unwanted cell populations, and trypsin. Steps 4 and 5 are repeated as many times as needed for the targeted dose or to freeze-down a cell bank (MCB/WCB strategy; which is an off-the-shelf product approach).
- Step 6: Cell concentration.
- Step 7: Resuspension of the cells in formulation buffer.
- Step 8: Filling of the cell suspension in the primary container (vial or bag) and labeling of the primary container. This is considered the drug product (DP).
- Step 9: For some products, the cells are immediately shipped by a qualified courier to the side of administration after step 8. In such cases, the hospital should be at a short distance, as the product cells are generally stable for hours to a couple of days at room temperature or at 2–8 °C (short-term storage; nonoff-the-shelf product). To allow for time between product manufacture plus quality control (QC) testing plus the release of the DP and administration, and to allow for easy shipment to distant hospi-

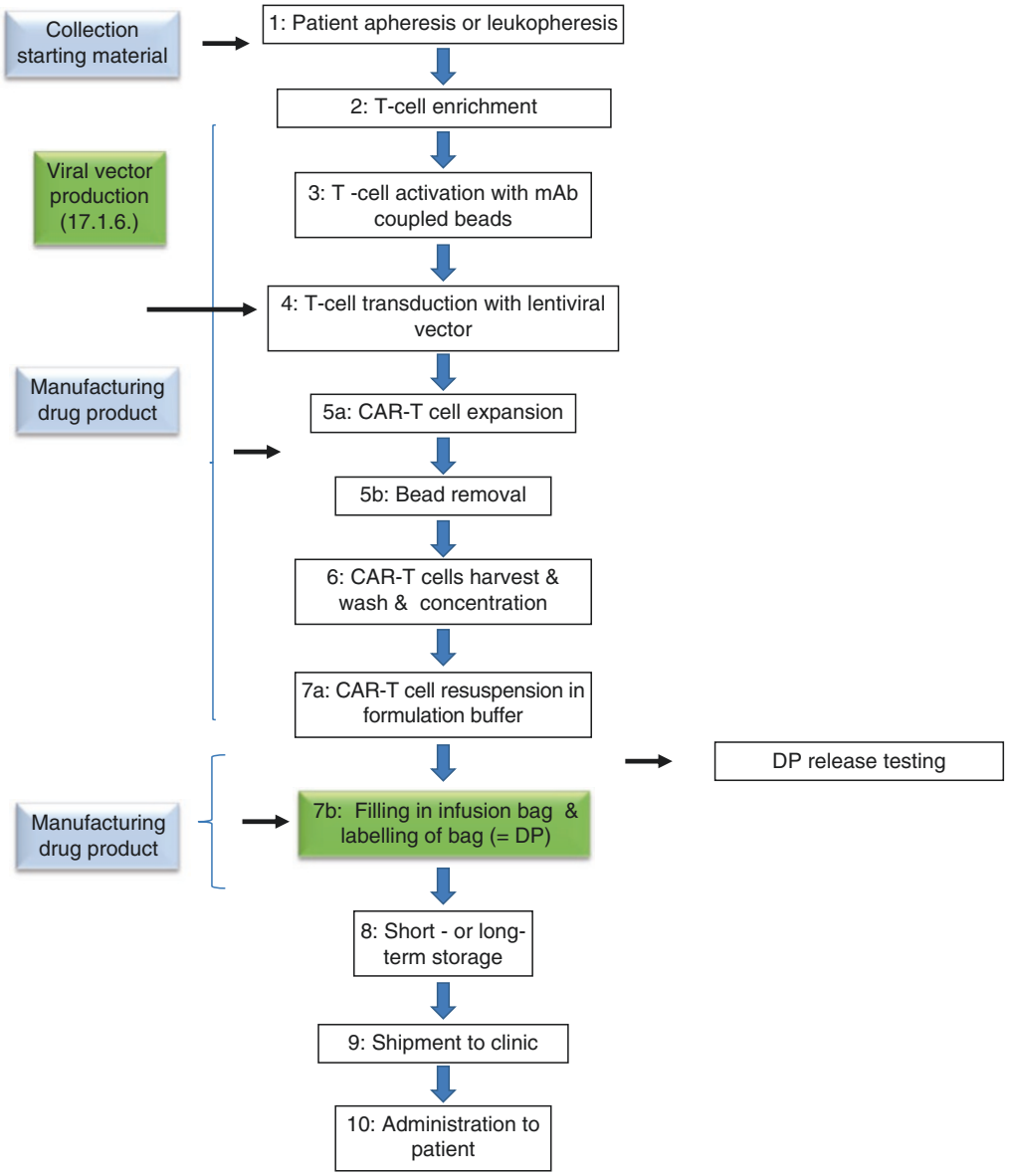

Fig. 14.33 Flow diagram of a CAR-T cell product manufacturing process. At the hospital white blood cells are harvested by leukapheresis (1). The starting material is shipped to the manufacturing facility for enrichment of the wanted T-cell populations (2); T-cell activation (3); transduction (genetic modification) of the T-cells with the lentiviral vector encoding the CAR genetic information (4). Thereafter, transduced cells (CAR-T cells) are ex vivo expanded (5a) and purified via bead removal (5b). Cells are harvested, washed, and concentrated (6); cells are resuspended in formulation buffer (7a) and filled in the primary container (7b), which is labelled. This is considered the drug product. The product is stored (8) and thereafter shipped to the clinic (9). Prior to administration via IV infusion of the CAR-T cells at the hospital (10), the patient is pre-conditioned with chemotherapeutic medicines. Except steps 1 and 10, which take place at the hospital, all other steps take place at a manufacturing facility under GMP conditions. QC testing occurs between steps 1–2 (control of the starting material), in-process (steps 2–7a), and on the final drug product (step 7b)

tals, the product is stored and shipped frozen, often in the vapor phase of liquid nitrogen at < −120 °C (long-term storage).

- Step 10: Shipment of the DP to the clinical site.
- Step 11: Administration to the patient systemically (IV infusion) or locally with/without the use of a surgical procedure.

Manufacturing of CAR-T Product

The manufacturing of genetically modified T cells is a multi-step process with slight modifications for each specific product (Fig. 14.33):

- Step 1: Harvest of blood cells by apheresis (whole blood collection) (Fig. 14.35)) or leukapheresis (collection of

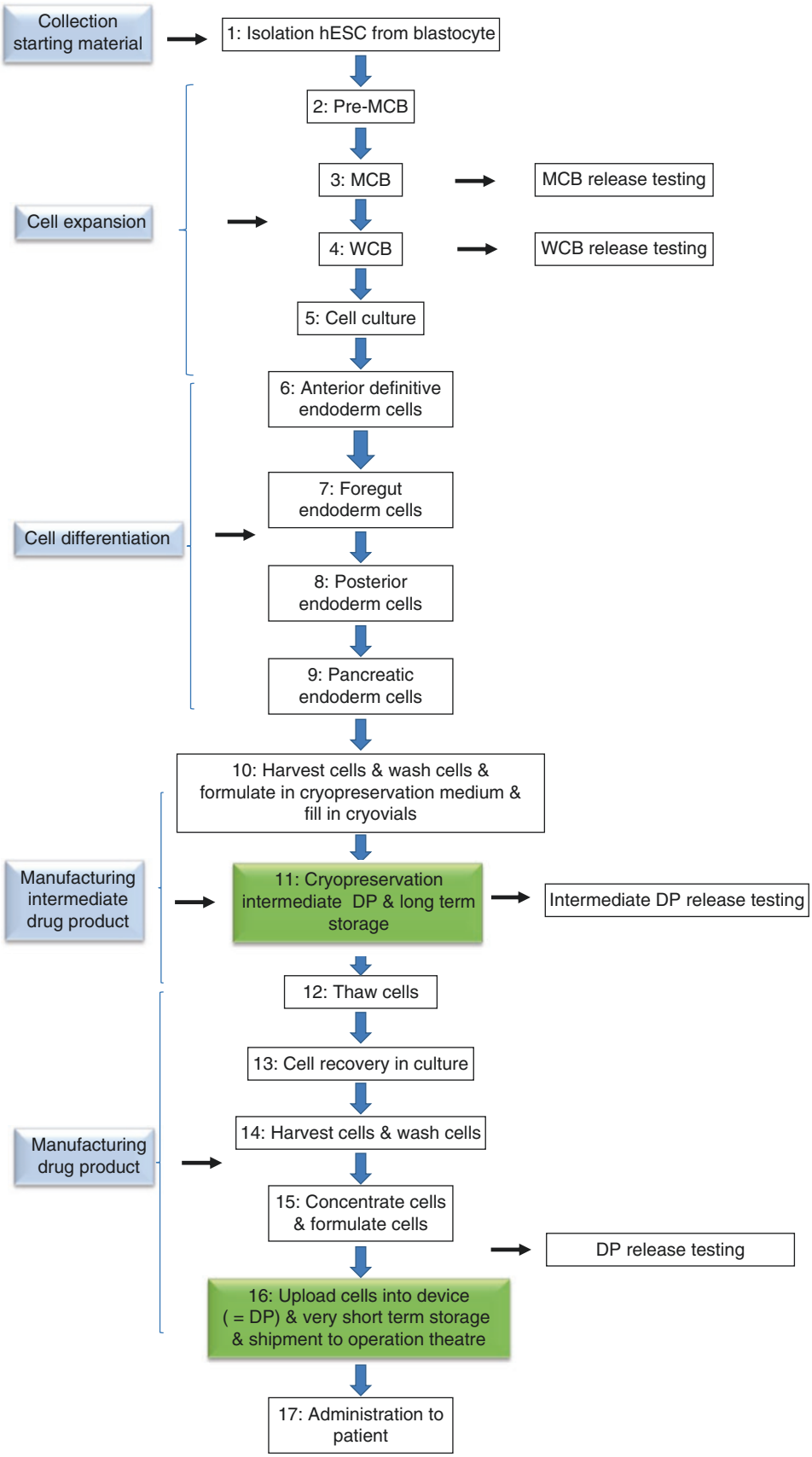

Fig. 14.34 Flow diagram of a hESC-derived combination product manufacturing process to treat DM type I

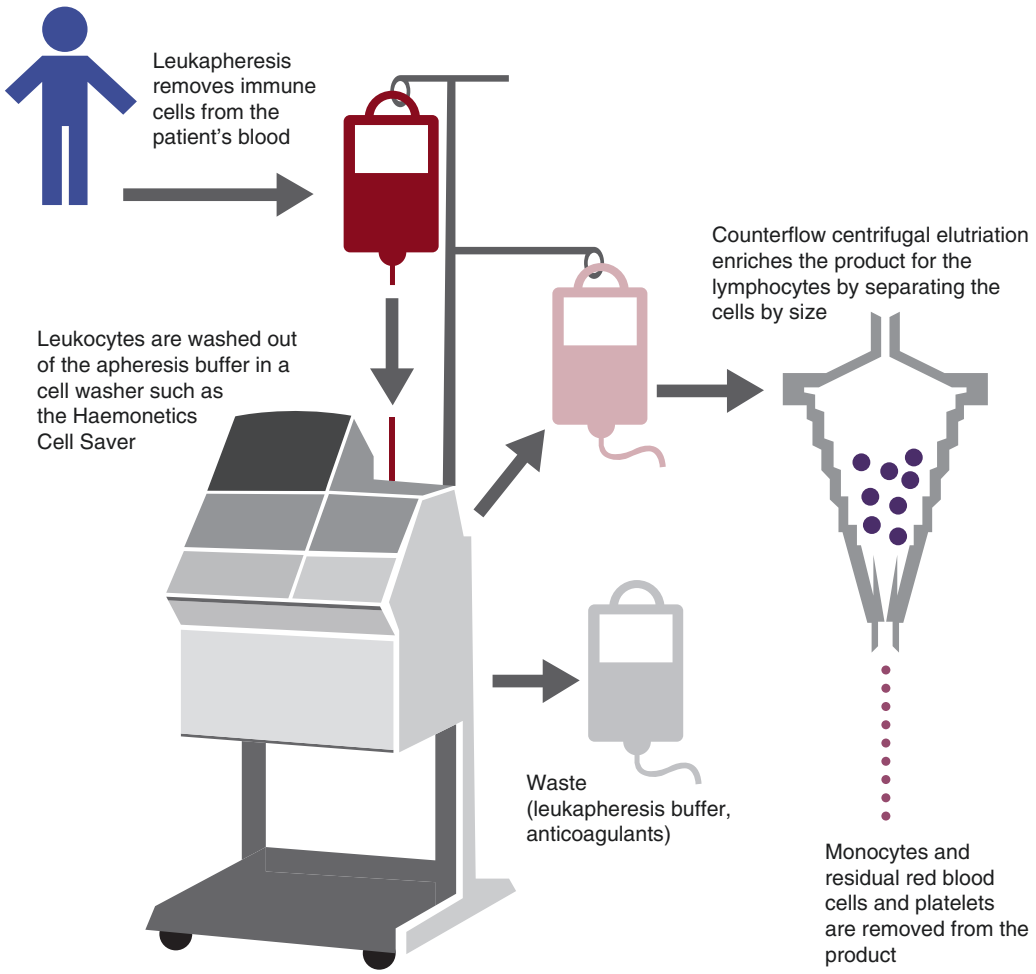

Fig. 14.35 Example of a leukapheresis system, which collects lymphocytes from the donor's peripheral blood, reprinted with permission (Levine et al. 2017)

leukocytes) from the patient (autologous cell source). The so-called "starting material" is shipped either "fresh," i.e., at room temperature or at 2–8 °C, or "frozen" (≤−80 °C) to the GMP manufacturing site. The patient is tested for specific human viruses prior to the donation of the starting material.

- Step 2: From this starting material, lymphocytes can be enriched either by counter-flow centrifugal elutriation or by subset selection according to the cellular phenotype.
- Step 3: The enriched lymphocyte population is placed in culture and stimulated with bead-based artificial antigen-presenting cells, e.g., magnetic beads, coupled with mAbs.
- Step 4: The viral vector is added to transduce the genetic insert (CAR) into the T cells.
- Step 5: The cell culture is expanded in a bioreactor for several days until sufficient numbers of CAR-T cells are obtained for dosing and QC testing. A magnet removes the beads from step 3 as they are considered a process impurity.
- Step 6: The T cells are harvested, washed, and concentrated.
- Step 7: The cells are resuspended in the final product formulation buffer (7a) and filled in the primary container (infusion bag or vial). This is the so-called "DP" (7b). Samples are taken for quality control testing.
- Step 8: For some products, the cells are immediately shipped by a qualified courier to the side of administration after step 7. In such cases, the hospital should be at a short distance, as the product cells are generally stable for hours to a couple of days at room temperature or at 2–8 °C (short-term storage). To allow for time between product manufacture plus QC testing plus the release of the DP and administration, and to allow for easy shipment to distant hospitals, the product is stored and shipped frozen, often in the vapor phase of liquid nitrogen at <−120 °C (long-term storage).
- Step 9: See step 10, manufacturing of MSC product
- Step 10: At the site of administration, the product is either administered directly to the patient or first thawed and

sometimes washed to remove certain excipients such as dimethyl sulfoxide (DMSO) and then administered, often via IV infusion.

The chain of the identity of the entire process, from leukapheresis to infusion and throughout all manufacturing steps and vice versa, i.e., from donor to recipient and from the recipient to donor, is controlled by a computer-based system to ensure the product's identity and product traceability.

Manufacturing of hESC Product

The manufacturing of an hESC-derived combination product (cells in device) to treat DM type I is a multi-step process with expansion and complex differentiation steps, with slight modifications for each specific product (Fig. 14.34):

- Step 1: Isolation of the starting material (hESCs) via the inner cell mass extraction. This procedure can only take place after informed consent from the parent(s) and testing of the mother's blood for specific human viruses. In addition, this step does not occur at a manufacturing facility under GMP but at an accredited tissue establishment, which is often a hospital.
- Step 2: Production of the pre-MCB by hESC culture initiation, cell expansion, cell wash, cell harvest, formulation of the cells in cryogenic medium, fill in a vial, and storage under cryogenic conditions in the vapor phase of liquid nitrogen.
- Step 3: Production of the MCB from a pre-MCB. A pre-MCB vial is thawed, and cells are cultured and expanded as described under "step 2," followed by release testing of the MCB.
- Step 4: Production of a WCB from the MCB (see step 3) and release testing of the WCB.
- Step 5: A WCB vial is thawed, and cells are expanded to obtain the required cell number for cell differentiation. Steps 2 through 5 take a couple of weeks.
- Step 6: Differentiation of undifferentiated hESCs into anterior definitive endoderm cells by adding specific growth factors and other factors to the culture medium. This step takes about 2 days.
- Step 7: Differentiation of anterior definitive endoderm cells into foregut endoderm cells by adding specific growth factors and other factors to the culture medium. This step takes about 3 days.
- Step 8: Differentiation of foregut endoderm cells into posterior foregut cells by adding specific growth factors and other factors to the culture medium. This step takes about 3 days.
- Step 9: Differentiation of posterior foregut cells into pancreatic endoderm cells by adding specific growth factors and other factors to the culture medium. This step takes about 4 days.
- Step 10: Pancreatic endoderm cells are harvested, washed, resuspended in a cryo-preservation medium, and filled in cryovials. The cryovials are labeled. This is considered the "intermediate DP."
- Step 11: The intermediate DP is cryopreserved in the vapor phase of liquid nitrogen at < -120 °C (long-term storage) and extensively QC tested prior to the release of the intermediate DP.
- Step 12: Intermediate DP cryovials are thawed. In case steps 2 through 11 take place at a GMP facility on long distance from the clinical site where the drug product will be administered to the patient, the cryopreserved intermediate DP is shipped frozen to a GMP facility, often the hospital pharmacy, for preparation of the final drug product.
- Step 13: Intermediate DP cells are recovered from the freezing and thawing steps by placing them in culture for another 3–4 days.
- Step 14: The recovered cells are harvested and washed to remove dead cells and culture medium.
- Step 15: Cells are concentrated and formulated in a buffer.
- Step 16: Cells are uploaded into the immune-protective device using a loading device. The pancreatic prebeta cells in the device are considered the DP. Limited QC release testing is performed on the DP.
- Step 17: The device is administered to the patient via a surgical procedure.

Key Factors for a Successful Manufacturing Process

To consistently manufacture advanced therapies at a large-scale, automated manufacturing processes as well as the implementation of functionally closed systems are key success factors for the following reasons: (1) lower the risk of viral and bacterial contamination during manual and open-process steps; (2) decrease costs associated with manual handlings; (3) improve product consistency; (4) shorten production times. Other key factors for success are logistics around the manufacturing, supply chain of the product, and the cost of goods. Particularly animal and human-derived raw materials, for example, growth factors, fetal bovine serum (FBS), antibody-coupled beads, and viral vectors, are very expensive. Considering the high cost and increased risk of validating sterilization cycles of multiple-use bioreactors, these closed-processes for advanced therapies utilize single-use, disposable bioreactors, mimicking current recombinant protein platform approaches (see Chap. 4). Despite some progress made in this field, there remains a requirement for a better understanding of potential manufacturing platforms and how they can be best utilized for advanced therapies,

taking the variety of cell and tissue types and clinical applications into account.

Viral Vector Production for Ex Vivo Gene Modification of Cells

Recombinant viral vectors, e.g., retroviruses like lentiviruses (cf. section on Viral Vectors in this chapter and Fig. 14.21), are produced by transfecting packaging cells, cultured with three to four plasmids that encode viral structural proteins, e.g., GAG, POL, Vesicular stomatitis virus (VSV)-G, and REV; the so-called packaging plasmids, and the plasmid encoding the therapeutic gene of interest, e.g., CAR, ADA-SCID; the so-called transfer plasmid. The transfer plasmid encoding the therapeutic gene contains the regulatory sequences that control its expression and a packaging sequence that enables its recognition. Within the packaging cell, e.g., the human embryonic kidney (HEK) 293 cell line, the RNA transcribed from the plasmid encoding the therapeutic gene is recognized by the viral proteins that assemble around it. The recombinant virus is then transported to the plasma membrane of the packaging cell that expresses viral envelope proteins (VSV-G). During budding, the virus acquires the lipid bilayer from the packaging cell surface and incorporates the envelope proteins. The viral vector particles are released from the cells cultured as adherent cells in culture flasks into the cell culture medium. The above-described steps are considered the upstream processing (USP) steps. The virus particles are subsequently harvested from the medium, formulated in a buffer, and filled in the primary container. These production steps are considered the downstream processing (DSP) steps (Morenweiser 2005). DSP steps applied for viral vector production are traditionally used in the biotechnology industry to manufacture recombinant proteins. These are membrane-based (filtration/clarification, concentration/diafiltration using tangential flow filtration, membrane-based chromatography) and chromatography-based (ion-exchange chromatography, affinity chromatography, and size exclusion chromatography) process steps. The combination of these different process steps is variable; in some cases, different purification principles are used for the same purpose. Furthermore, a benzonase/DNase treatment for the degradation of contaminating DNA from the packaging cells is either included in the USP or DSP part of the manufacturing process. Subsequently, the purified virus particles are formulated in a buffer, filled in the primary container, stored frozen, and tested until further use for transduction of the cells to make a genetically modified cell therapy product (Wright 2008). Figure 14.36 provides a schematic overview of the entire viral vector material manufacturing process used to produce a genetically modified cell therapy product. For the production of a viral vector product for in vivo gene therapy (see later in this chapter), the production process is identical.

Excipients

Common excipients used in the formulation of advanced therapies are presented in Table 14.15. Most of these excipients overlap with those used in therapeutic protein products. However, KCl, $MgCl_2$, nucleosides, FBS, and DMSO are not found in therapeutic protein drug products.

Table 14.16 provides an overview of a few commercially available advanced therapies with their formulation and shelf-life

Add to legend under [c]: DMEM = Eagle's minimal essential medium...and then the rest of the text

Primary Container

Generally, two types of containers are used for cell-based products: vials (small volume, low dose) and infusion bags (higher volume and dose), as shown in Fig. 14.37. However, tissue-based products often have a nonstandard container for storage and shipment.

Storage and Shipment

Stability of the starting material (cells or tissue and viral vector) and DP are an important element for the successful production, storage, shipment, and administration of advanced therapies. Starting materials and DPs either have a very short shelf-life of hours–days and are stored and transported at 2–8 °C or at room temperature or have a longer shelf-life (months–years) and are stored and shipped frozen (cryopreserved in the vapor phase of liquid nitrogen at <−120 °C or in a −80 °C or −150 °C freezer).

Manufacturing Model: Scale–Up Versus Scale-Out

Broadly speaking, there are two paradigms in advanced therapy manufacture: off-the-shelf (always allogeneic source of cells/tissue) and patient-specific (commonly autologous source of starting materials, but sometimes allogeneic) DPs. Off-the-shelf products represent a business model akin to current biopharmaceuticals, where one batch can be manufactured to treat multiple patients. This allows for increased economies of scale, which drives down the per-dose cost of the final product. This means that there is a wealth of engineering and process knowledge and technologies that can be leveraged to support the manufacture of off-the-shelf advanced therapies at an increasing scale.

However, scale-up is not just about making the reactor grow the cells bigger. Conventional scale-up bioprocesses typically use cells to produce therapeutic agents (e.g., mAbs),

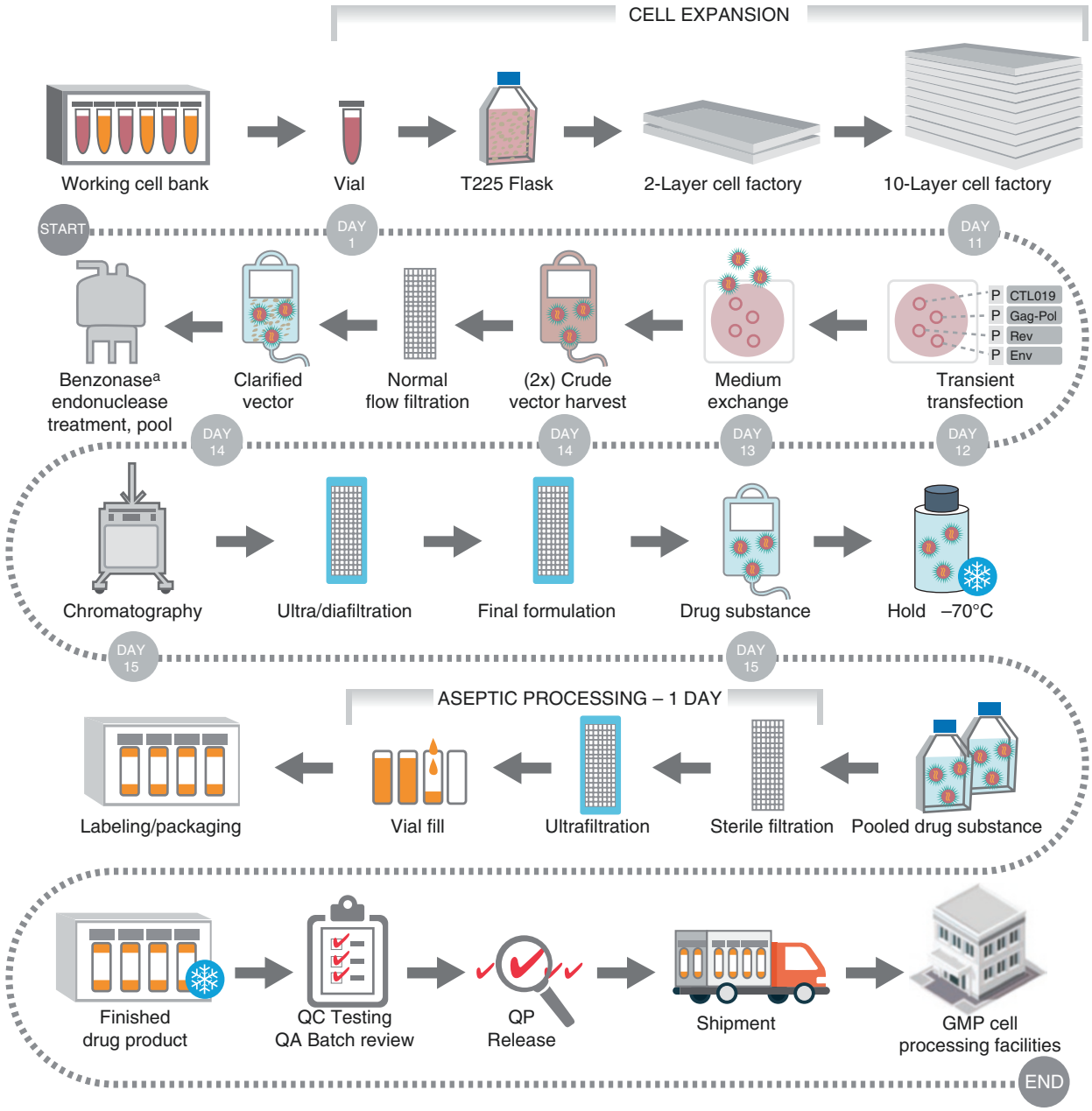

Fig. 14.36 Schematic overview of a lentiviral vector manufacturing process. The produced viral vector is used as starting material for the genetic modification of T-cells in the manufacture of a CAR-T product, reprinted with permission (Levine et al. 2017). A similar production approach is taken for other ex vivo gene therapy as well as in vivo gene therapy products (cf. Chap. 16). QC = quality control, QP = qualified person, QA = quality assurance

which can then be isolated and purified without the need to recover the cell. For the manufacture of advanced therapies, where the cells/tissue culture is the product of interest, retention of cell viability, phenotype, and function to assure quality, is of primary importance in order to preserve product safety and efficacy. As the number of cells increases during expansion, this can become increasingly challenging, as the greater cell numbers lead to an increased chance of inhomogeneity of culture and hence of cellular performance being altered. This means that the desired quality of the cells/tissue must be maintained through the entire manufacturing process, including the harvest and DSP, storage, shipment, and delivery to the patient. This will require the development of scalable harvesting, DSP, and formulation technologies to cope with the large batch size produced.

Patient-specific advanced therapies offer a new challenge for process scalability, where the manufacturing process must be scaled-out in order to produce one batch for each patient (Fig. 14.38). This introduces the concept of "personalized medicine" (see Chap. 9), where the cost of production

Table 14.15 Examples of excipients used in the formulation of advanced therapy products

Excipients class	Function	Example
Buffer	pH stabilizer	TRIS, histidine, Na-acetate
Salt	Stabilizer	NaCl, KCl, $MgCl_2$
Antioxidant	Prevent oxidation	Methionine
Sugar	Stabilizer, cryoprotectant tonicity modifier	Mannitol, trehalose, sucrose, glucose
Polyol	Collapse temperature modifier	Dextran (low and high molecular weight)
Nucleoside	Stabilizer	Adenosine, guanosine
Protein	Stabilizer, preservative	Fetal bovine serum, human serum albumin
Organic solvent	Stabilizer, cryoprotectant solvent	Glycerol, ethylene glycol, DMSO

Table 14.16 Examples of approved advanced therapies, their formulation, and shelf-lives

Product	Shell-life and storage condition	Composition (active substance)	Excipients/mixtures
Provenge® Suspensbn of cells for IV infusion	18 h at 2–8 °C	≥50 × 10^6 autologous $CD54^+$ cells/250 ml activated with PAP-GM-CSP[a]	Lactated Ringer's solution (NaCl, $NaC_3H_5O_3$, KCl, $CaCl_2$)
ChondroCelect® Suspension of cells for implantation	48 h at 15–35 °C	4 × 10^5 autologous human cartilage cells/ 0.4 ml	DMEM[b]
MACI® Implantation matrix plus cells in solution for implantation	6 days at ≤37 °C and keep out of fridge	0.5 × 10^5 to 1 × 10^6 autologous cultured chondrocytes/cm^2 porcine derived type I/III collagen membrane	DMEM, HEPES[c] adjusted for pH with HCl or NaOH and osmality with NaCl
Kymriah® Suspension of calls for IV infusion	9 months at ≤–120 °C in the vapor phase of liquid nitrogen	2 × 10^5–2.5 × 10^5 autologous CAR-positive viable T cells	Plasmalyte-A[d], glucose/NaCl, human serum albumin, dextran 40-low molecular weight/glucose, DMSO

[a] Prostatic acid phosphatase granulocyte-macrophage colony-stimulating factor
[b] Calcium Chloride anhydrous, Ferric Nitrate·$9H_2O$, Potassium Chloride, Magnesium Sulphate anhydrous, Sodium Chloride, Sodium Bicarbonate, Potassium Phosphate Monobasic·H_2O, D-Glucose, L-Arginine. HCl, L-Cystine·2HCl, L-Glutamine, Glycine, L-Histidine·HCl·H_2O, L-Isoleucine. L-Leucine, L-Lysine·HCl, L-Methionine, L-Phenylalanine. L-Serine. L-Threonine, L-Tryptophan, L-Tyrosin·2Na·$2H_2O$, L-Valine, D-Calcium Pantothenate, Choline Chloride, Folic Acid I-Inositol, Niacinamide, Riboflavin·Thiamine·HCl, Pyridoxine·HCl
[c] 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium, *DMEM* Eagle's minimal essential medium
[d] Plasmalyte-A sodium chloride: 5.26 g/l potassium chloride: 0.37 g/l magnesium chloride hexahydrate: 0.30 g/l sodium acetate trihydrate

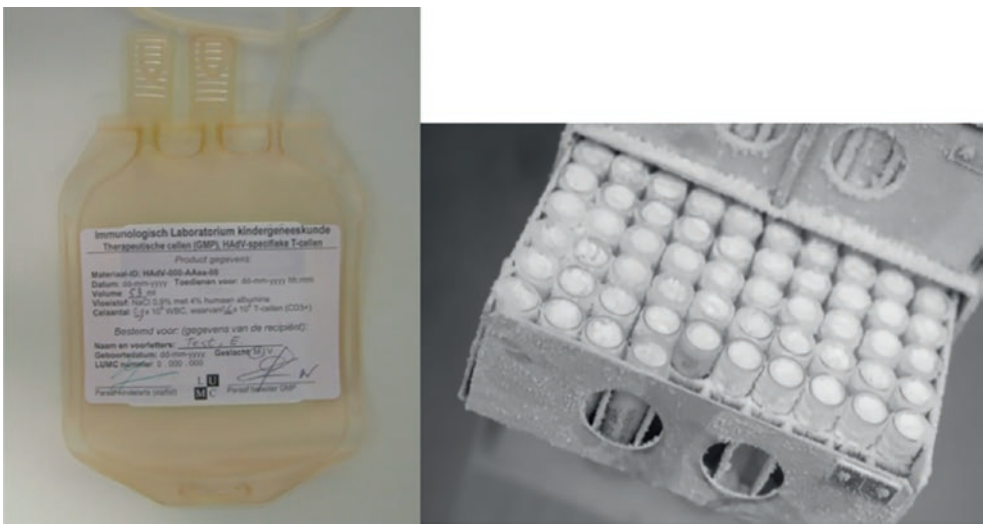

Fig. 14.37 Examples of primary containers for the storage and transport of advanced therapies. Left photo: infusion bag; right photo: cryovials in box to allow for storage in the vapor phase of liquid nitrogen (courtesy of M. de Haan)

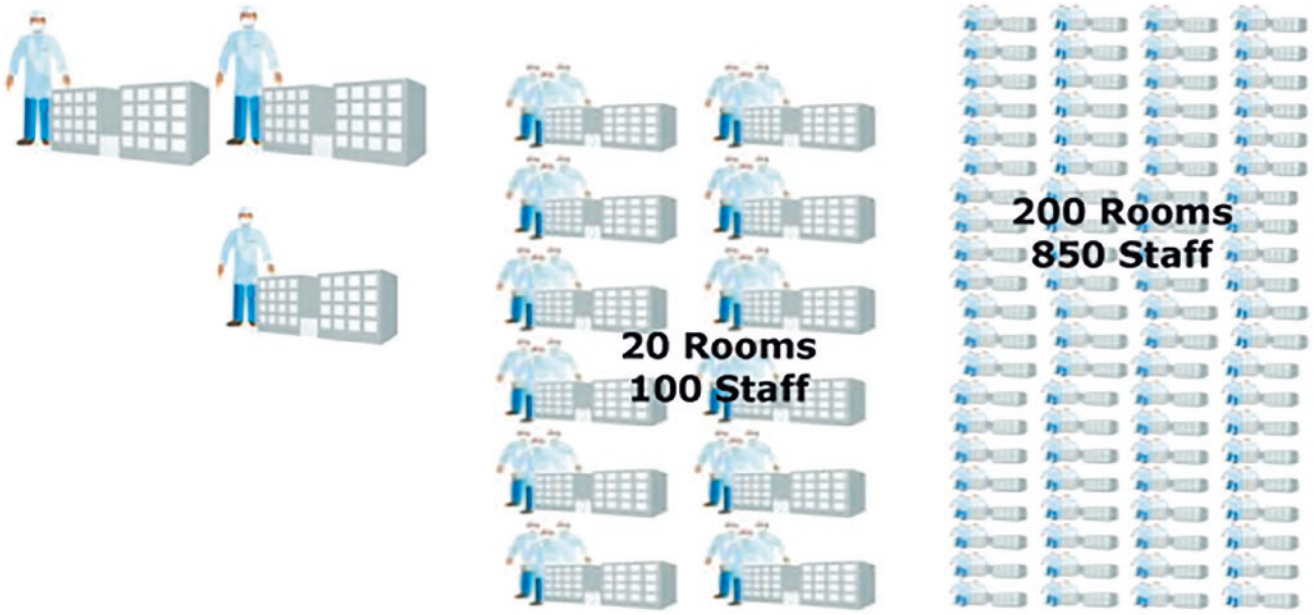

Fig. 14.38 Scale-out of a labor intensive manual process

per batch cannot be reduced by exploiting an increasing economy of scale by simply producing a larger batch. Reducing the cost of these patient-specific cell- and tissue-based products must therefore be achieved by advances in engineering and manufacturing technology, reducing the number of complex, labor-intensive, and open-process steps that are commonplace in the manufacture of these products at research labs. The developments of closed and automated processes as well as process simplification, are key factors for commercial success as this will allow multiple batches to be produced in parallel (scale-out), with reduced burden of oversight by highly-trained scientists. These new processes must be GMP-compliant and closed for sterility.

Testing

As for any DP, cell- and tissue-based therapies are subject to detailed characterization. This involves the assessment of quality attributes, i.e., identity, purity and impurities, viability (Cadena-Herrera et al. 2015), bioactivity (potency; Bravery et al. 2013), safety, quantity, and general attributes, such as appearance, pH, morphology both of the cellular/tissue/vector starting material and the final DP, see Table 14.17. The latter includes QC testing to allow the release of the DP for administration. In addition, at different stages of production, in-process controls are performed to assess the quality and stability of the cells/tissue during manufacture. Finally, a subset of characterization tools is used to assess the stability of the starting material(s) and DP.

However, for a lot of autologous and some allogeneic DPs that are not "off-the-shelf," performing QC tests may be challenging due to the time constraints between manufacture and administration, i.e., the shelf-life of the drug product is hours–days. Moreover, for some autologous products, all the available cell/tissue material is needed for the dose. In such cases, product release may be justified by extensive process validation; in-process control testing and/or QC testing data becoming available after product administration. These approaches require a paradigm shift in the pharma world, where traditional products are only administered after extensive testing and batch release. Adequate QC of starting materials such as cells/ tissue biopsy and viral vectors is crucial as poor-quality starting material will affect the quality of the final product. Autologous or allogeneic cells/tissue can be very heterogeneous due to the inherent donor variability (age, sex, health status, medication), the variable number of cells other than the intended cells, and because the collected cells are not in a synchronized cell cycle. In addition, the origin of the cells, e.g., MSCs of bone marrow, adipose, and cord blood origin, may have a significant impact on the activity and phenotype of the cells after manufacture.

The challenge is that a lot of the techniques used for the characterization of this heterogeneous group of products are not sensitive methods; hence they are not able to pick-up subtle changes to the process and/or to the product.

For further reading oncell- and tissue-based product characterization, see BSI PAS 93:2011. For details on testing (lot release and additional characterization) of viral vectors for

Table 14.17 Examples of techniques applied for the analysis of different quality attributes of cell- and tissue-based therapies

Quality attribute	Explanation	Possible techniques applied
Identity	Distinguish the cellular active substance (s)/ tissue from unwanted cell population(s); donor specific test; sometimes a combination of tests	Flow cytometry; karyology, STR, FISH, CGH, microscopy, immunocytochemistry, electrochemiluminescence, protein array, microarray
Active substance purity	Number of viable cells with specific cell surface markers present/absent, unique for the active substance. Closely related to identity	Flow cytometry; ELISA; immunocytochemistry; electrochemiluminescence; protein ligation assay
Cellular (product) impurities	Dead cells (based on total and viable cell numbers); unwanted cell populations. Closely related to identity and purity	Flow cytometry; ELISA; electrochemiluminescence; MS
Process impurities	Depends or process and raw materials used. e.g. antibiotics, cytokines, growth factors, FBS, beads, viral vector starting material	– Cytokines, growth factors, FBS, TryPLESelect: ELISA – Beads: microscopic evaluation; – Antibiotics: LC-MS; – Viral vector: qPCR
Potency/bioactivity	Quantitative measure of relevant biologic function(s) based on the attributes that are linked to relevant in vivo biologic properties; often a combination of assays. Receptors, cellular metabolism, secreted proteins, migration of cells, (lack of) proliferation, differentiation potential, mRNA expression	ELISA; qPCR; Flow cytometry; cell migration in Dunn or Boyden chamber: protein array; LC; MS; animal modal (not quantitative), microarray
Viability and total cell count	Viability is a critical parameter and related to dose, purify and cellular impurities	Colorimetric assay (spectrophotometer), fluorescent assay (including flow cytometry), membrane integrity assay (e.g., trypan blue), microscope. Manual. semi-automated or automated equipment
Dose	Often number of total or viable cells per unit (mL, kg body weight); cm^2 of tissue	Total call count and viability techniques
Safety	Sterility, endotoxin, mycoplasma, human and animal viruses derived from starting material or raw materials, replication competent viral vector, chromosomal aberrations	Pharmacopoeial tests for sterility, mycoplasma. endotoxin-standard or rapid tests; chromosomal aberrations by karyology FISH, CGH
General attribute	Appearance, pH, osmolality, particles, cell/tissue morphology	Pharmacopoeial tests, microscope for morphology assessment

Some techniques are also used for starting material characterization
Flow cytometry technique is explained below; it can be used for intracellular and cell surface markers
STR short tandem repeat, *FISH* fluorescence in situ hybridization, *CGH* comparative genomic hybridization, *ELISA* enzyme-linked immuno sorbent assay; see Chap. 3 for details on this technique, *MS* mass spectrometry, *LC-MS* liquid chromatography-mass spectrometry; see Chap. 3 for detail on this technique, *qPCR* quantitative polymerase chain reaction; see Chap. 1 for details on PCR

ex vivo and in vivo gene therapy products, see Gombold et al. (2006a, b). Table 14.18 provides an overview of the QC testing panel for an MSC-derived and a CAR-T product.

Flow Cytometry

One of the key technologies in advanced therapy manufacturing is flow cytometry. It can be operated in a QC test environment and in the production of advanced therapies products (see next section). As this technique is not used regularly to characterize therapeutic proteins, it is not discussed in Chap. 3. Therefore, we pay attention to it in this chapter.

Flow cytometry assays may be used to assess cell- and tissue-based product identity, active substance purity, cellular impurity, viability, and potency testing. It is a powerful technique that allows for a specific measurement of cellular components on the cell surface, e.g., CD73, CD90, and CD105, to characterize MSCs, and intracellular components. It is also amenable to the measurement of soluble analyte(s) such as cytokines, released by the cells in the extracellular environment, e.g., upon cell activation.

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light (Fig. 14.39). The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or par-

Table 14.18 Example of QC testing panel for an MSC-derived cell based product and a CAR-T ex vivo gene therapy product

Quality attribute	MSC derived cell based product; allogeneic off-the-shelf (1 batch of multiple vials/bags for multiple patients)	CAR-T ex vivo gene therapy product; autologous (1 batch of 1 infusion bag for 1 patient)
Identity	$CD73^+$, $CD90^+$, $CD105^+$, $HLA\text{-}DR^-$, $CD3^-$, CD45- cells by flow cytometry	CAR expression by qPCR
Viability by manual or automated cell count	Number of total cells	Number of total cells
	Number of viable cells	Number of viable cells
	Percentage of viable cells	Percentage of viable cells
Purity by flow cytometry (% of viable cells with a certain CD-marker profile)	Percentage of $CD73^+$, $CD90^+$, $CD105^+$. $7\text{-}AAD^-$ cells by flow cytometry	Percentage of viable T cells
		Transduction efficiency by CAR q-PCR
Product = cellular impurities (dead cells and unwanted cell populations) by flow cytometry	Percentages of 7-AAD+ (dead cells), $CD3^+$ (T cells), $CD45^+$ (lymphocytes). $CD34^+$ (HSCs and endothelial cells), $CD14^+$ (monocytes), $CD19^+$ (B cells)	Percentages of red blood cells, granulocytes, dead cells, $CD19^+$ B cells
Process impurities	Residual bovine serum albumin (BSA) by ELISA	Residual antibody conjugated beads (CD3/CD28)
	Residual TryPLESelect by ELISA	BSA by ELISA
	Residual antibiotic by liquid chromatography–mass spectrometry	Residual VSV-G DNA by qPCR-derived from viral vector
Potency	CD marker expression (e.g., adhesion molecules) upon immune activation by flow cytometry	Determination of CAR expression by flow cytometry
		Release of interferon-gamma in response to CD19-expressing target cells
Safety	Sterility	Sterility
	Bacterial endotoxins	Endotoxin
	Mycoplasma	Mycoplasma
	Karyology	PCR-based replication competent lentivirus assay
	Human viral testing; test for the presence of inapparent virus; in-vitro assay for the presence of viral contaminants	N.A.
Dose (calculated)	a–b × 10^6 viable $CD73^+$ $CD90^+$, $CD105^+$, $7\text{-}AAD^-$ cells/ml	a–b × 10^6 $CD19^+$ T cells/kg body weight
General attribute	pH	pH
	Osmolality	Osmolality
	Appearance of primary container and content	Appearance of primary container and content
	Content uniformity	N.A.
	Extractable volume from the vial	N.A.

ticle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

- The fluidics system transports single particles (cells) in a stream to the laser beam for interrogation.
- The optics system consists of a light source, mostly lasers, to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. Light scattering or fluorescence emission from auto-fluorescence of the particle or from fluorophores, which are fluorescence labels, e.g., bound to specific antibodies, used to detect the expression of cellular molecules such as specific proteins or nucleic acids, provides information about the particle's properties. (1) Light that is scattered in the forward direction after interacting with a particle, typically up to 20° offset from the axis of the laser, is collected by a photomultiplier tube or photodiode and is known as the forward scatter (FSC) channel. This FSC measurement can estimate a particle's size, with larger particles refracting more light than smaller particles. (2) Light measured at a 90° angle to the excitation line is called side scatter (SSC). The SSC can provide information about the relative complexity, e.g., granularity and internal structures, of a cell or particle. However, as with forward scatter, this can depend on various factors. Both FSC and SSC are

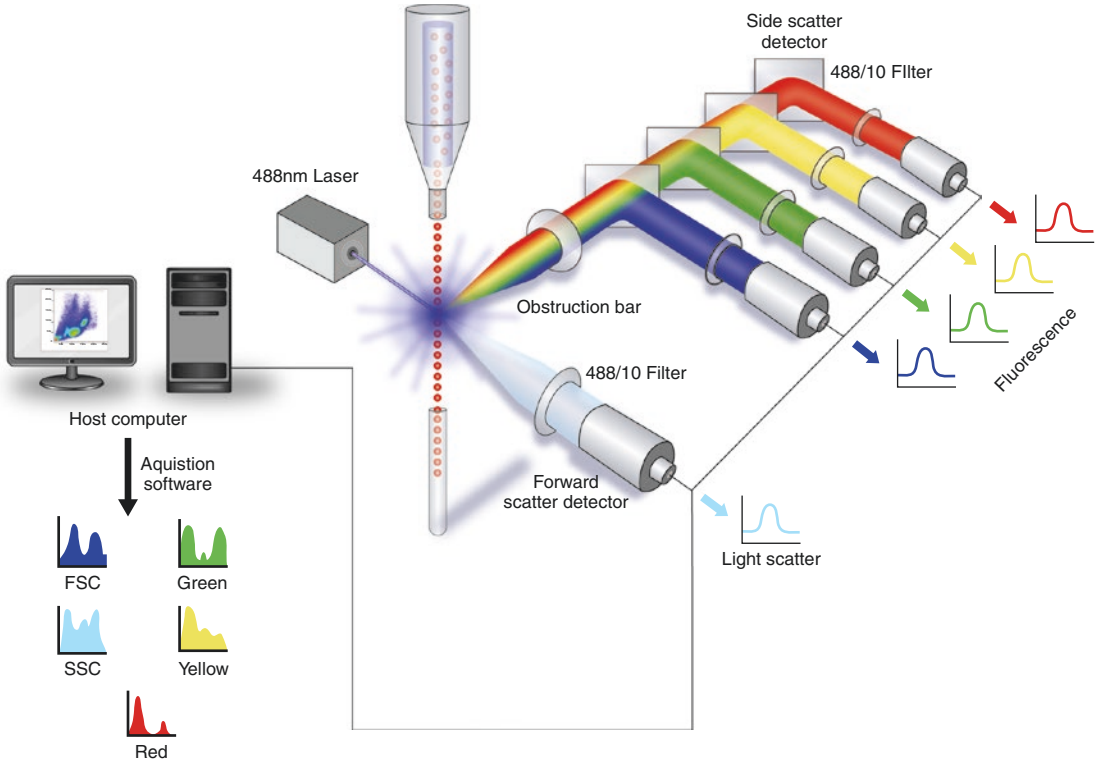

Fig. 14.39 Schematic view of a flow cytometer. Scattered and emitted light signals are converted to electronic pulses, adapted from ThermoFisher Scientific. http://www.thermofisher.com/nl/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecularprobes-school-of-fluorescence/flow-cytometry-basics/flow-cytometry-fundamentals/how-flow-cytometer-works.html#overview

unique for every particle, and a combination of the two can be used to roughly differentiate cell types in a heterogeneous population such as blood or bone marrow aspirate. However, this scatter information and cell typing depend on the sample type and the quality of sample preparation, so fluorescent labeling is generally required to obtain more detailed information.

– The electronics system converts the detected light signals into electronic signals that the computer can process.
– In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2 to 150 μm in size is suitable for analysis. Cells from solid tissue must be desegregated into single cells before analysis. The portion of the fluid stream where particles are located is called the sample core. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecule present on the particle fluoresces. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. Readouts are collected on each particle or single event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations of cells within the sample (see Fig. 14.40).

Fluorescence-Activated Cell Sorting (FACS)

Flow cytometry techniques can also be used to sort specific cell (sub) populations, e.g., to increase product yield and/or reduce the amount of unwanted cell populations, which are considered impurities. A FACS machine provides the ability to separate cells identified by flow cytometry. Droplet-based cell sorters first analyze the particles but also have hardware that can generate droplets and deflect or direct wanted particles into a collection tube. Cell dispersions are often purified based on surface markers such as CD34+ in HSCs or on their

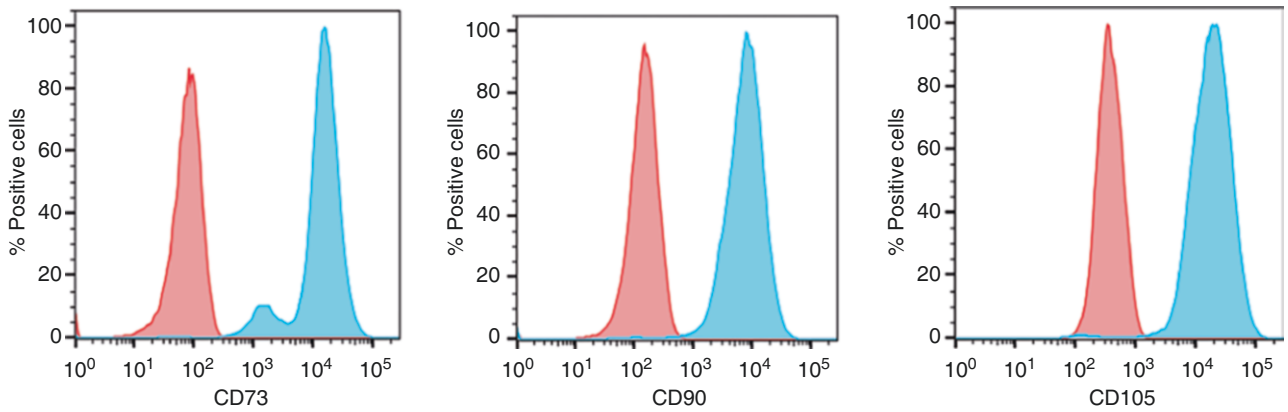

Fig. 14.40 Flow cytometry histograms of MSC product cells. Flow cytometric analysis of MSC product cells against three defined MSC markers (CD73, CD90, and CD105) show that these cells are of mesenchymal cell phenotype. On the X-axis the density of the respective cell surface marker molecule is shown. A single peak is observed for each of the markers tested (blue peak at the right side of each histogram), indicating a single population of cells. The red peak at the left side of each histogram represents the isotype control staining. Courtesy of M. van Pel

viability. Common uses of cell sorting include identifying and isolating cell populations or single cells followed by subsequent downstream applications where DNA, protein, or cellular function is investigated.

Improvements in Testing Strategies Needed

Developing robust, sensitive, rapid, and in-line analytical testing and characterization tools will be required as cell/tissue and viral vector processing platforms continue to evolve. Significant improvements are needed to establish next-generation analytics for (in-process) QC, stability, and additional characterization testing to assess the quality attributes of starting materials, intermediates, and advanced therapy products. Improvements are also to be made in the field of in-line and online testing of cell culture conditions, e.g., pH, morphology, and viability. Reducing the sampling frequency, technical complexity, amount of sample needed, and labor intensiveness of testing are especially critical for a nonoff-the-shelf autologous ex vivo gene therapy product. This contrasts with traditional biopharmaceuticals, where a single batch of QC tested products may treat hundreds or thousands of patients. Cell processing automation will also be enabled through the development of high throughput in-process and release assays providing results in a very short time frame (minutes–hours). Advanced cell/tissue characterization techniques based on nanofluidics, transcriptomics, and proteomics, and next-generation sequencing techniques may allow a better understanding of what happens to desired cell population(s)/tissue once they are processed and before patient administration (see Chap. 9 for more details on "-omics"), both in the cytosol as well as in the extracellular environment. Examples are changes in intracellular genetic profiles and patterns within the micro-RNA and exosome pools secreted into the culture medium by the cells.

Different advanced therapy technologies are currently at different translation stages and have their particular manufacturing and testing challenges, as summarized in Table 14.19.

Table 14.19 Development stage manufacturing and testing challenges for different advanced therapy technologies, adapted from Mount et al. (2015)

Technologies	Development stage of the field	Current manufacturing technologies	Manufacturing and testing challenges
(a) Somatic cell technologies	Many products in early clinical development phase; few products approved, e.g., Alofisel	Manual process with open handling steps; automated multi-planar flasks and stack systems; micro-carriers in disposable stirred tank systems; hollow fiber growth systems; membrane and contraflow centrifugation systems	Scale-up and control of large scale batches. Recovery of cells from micro-carriers. DSP: Large volume handling, primary container filling at scale using enclosed technologies. Relevant potency assays lacking
(b) Cell immortalization technologies	One product in early clinical development	CompacT Select[a] fully automated and programmable scalable cell culture platform consisting of a robot arm that can access T175 flask or multi-well plate incubator. Standard cell culture activities, such as passage or media change, are conducted and controlled with no manual intervention	Similar to protein manufacturing platform technologies
(c) Ex vivo gene modification of cells using viral vector technologies	Mainly small trials in early and late clinical development phase (gene modified autologous T-cells and HSCs); few products approved, e.g., Strimvelis and Kymriah	Manual processes often not fully enclosed using static bags, gas-permeable pots + lateral movement bioreactors (wave bags) for higher cell yield. Positive or negative cell selection process steps often used. High cell purity becoming possible with sterile cell sorter	Adapting systems to deal with variation in quality and amount of incoming starting material. Lack of product stability pressuring manufacturing and distribution model. Lack of fast QC assays. Low transduction efficiency with non-replicating viral vectors. Enclosed and automated manufacturing systems are becoming available for the entire process (e.g., prodigy)
(d) Cell plasticity technologies	Mainly pre-clinical phase with few ESC and iPSC-derived FIH trials	Current processes are extremely 'manual' and rely on small scale cell culture and harvest technologies. High risk processes with extensive process and product characterization testing to assess product quality, safety, and efficacy	A two-tier banking strategy (MCB/WCB) scale-up process of pluripotent cells prior to differentiation steps needed. Dynamic cell culture systems to expand PSC numbers. Robotic scale-out of current plate-based iPSC technology is also being explored
(e) 3D-technologies	Mainly pre-clinical phase with few FIH trials	A complex manufacturing interplay between (bio)materials, scaffolds, cells, and biological coatings. Incorporates decellularization/recellularization tissue-based products such as trachea, esophagus, and veins	Enclosed bioreactors to control cell and material interface. Improved stability and delivery systems. Robust product quality to ensure large clinical application

[a] Thomas et al. (2009)

Other Aspects of Advanced Therapies

Regulatory Bodies Involved in Regulating Advanced Therapies in Europe

In Europe, the responsibility for regulating transplant products according to the public health legislation lies with the national Competent Authority for tissues and cells in each member state. ATMPs, in contrast, are regulated by pharmaceutical legislation. Hence, marketing approval must be obtained before marketing an ATMP through the centralized procedure, as for any other biological medicinal product. The scientific evaluation of these products is led by a specialized committee within the EMA (the Committee for Advanced Therapies "CAT"). The CAT drafts an opinion for the Committee for Medicinal Products for Human Use (CHMP), which is responsible for providing a second scientific opinion. Based on a positive CHMP opinion, the approval of a marketing authorization application (MAA) is granted by the European Commission. Clinical trials involving ATMPs are regulated and authorized the same manner as

Table 14.20 Regulatory agencies and applicable guidances for advanced therapies in the US and EU

Regulatory agency/institute	Link
EMA	https://www.ema.europa.eu/en/human-regulatory-overview/advanced-therapy-medicinal-products-overview
FDA	https://www.fda.gov/BiologicsBloodVaccines/default.htm
International Conference on Harmonisation (ICH)	https://www.ich.org/
British Standards Institution (BSI)	BSI PAS 83:2012, BSI PAS 84:2012, and BSI PAS 93:2011

other medicinal products, i.e., on a national level by the appropriate national competent authority (NCA).

Regulatory Bodies Involved in Regulating Advanced Therapies in the USA

The situation in the United States is simpler in that the FDA is responsible for both aspects of the legislation: the public health and pharmaceutical legislation. Within the FDA, the responsibility for the regulation of HCT/Ps and human gene therapy products lies with the Center for Biologics Evaluation and Research (CBER), both for clinical trials and marketing authorization. As of 2016, the CBER structure includes the Office of Blood Research and Review (OBRR), the Office of Vaccines Research and Review (OVRR), and the Office of Tissues and Advanced Therapies (OTAT), which was formerly known as the Office of Cellular, Tissues and Gene Therapies (OCTGT). To monitor activity, review data, and anticipate future needs, the FDA operates the Cellular, Tissue, and Gene Therapies Advisory Committee.

Regulatory Guidances

Links to the relevant regulatory bodies involved in advanced therapies in the EU and US, as well as applicable guidances, can be found in Table 14.20.

Stem Cell Tourism

The general interest in advanced therapies worldwide has allowed unregulated practice, particularly of cell-based products, to develop in some countries, i.e., "stem cell tourism." This is a major concern for many stakeholders in the field of ATMPs because treatments are being offered in the absence of a strong safety data package and any proven efficacy. In addition, there is suspicion that the products in use have been manufactured with insufficient attention to GMP, including quality control. Patients must be warned of the dangers of falling prey to unethical operations. An up-to-date source of information on private clinics and stem cell tourism is available at the website of the International Society for Stem Cell Research (www.isscr.org).

Concluding Remarks

Although progress has been made in the area of ATMPs, with about 80 products approved globally and 27 in the USA & EU for commercial use (see examples in Table 14.6) and many products in clinical development, this field was currently struggling with similar problems as the first recombinant proteins 20 years ago. Appropriate manufacturing platforms, supply chain models, healthcare systems, reimbursement models, and regulatory frameworks for these medicinal products need to be established by developers and other key stakeholders, while specific knowledge about quality (production and testing), safety, and efficacy of advanced therapies is steadily growing.

Self-Assessment Questions

Questions

1. What is the difference between embryonic and adult stem cells?
2. How is somatic cell nuclear transfer carried out, and what are the problems with this technique?
3. What are iPSCs, and why are they important?
4. What is the difference between in vivo gene therapy and ex vivo gene therapy?
5. Which disease areas are predominantly investigated clinically with ATMPs?
6. What problems could arise in the use of stem cell-derived products for clinical application?
7. What was the disease target for the first gene therapy clinical trial? What vector was selected for gene transfer?
8. Identify and describe five transcription regulatory elements discussed in the chapter.
9. Several clinical trials involve gene transfer for treating malignant glioma. One approach involves the use of a recombinant retrovirus expressing the HSV-tk transgene. Another involves the use of a recombinant adenovirus expressing the p53 transgene.
   (a) Which of the five current strategies to treat cancer by viral gene therapy does each of these trials employ? Describe the principle behind each strategy.

(b) List two advantages and two disadvantages associated with the vector used in each of these trials.
(c) Outline potential drawbacks to the use of each of these strategies for cancer therapy.
(d) What other approaches could have been selected to prevent the growth and spread of malignant tissue? Explain the principle behind each.

10. What is the purpose of the packaging cell line during the production of recombinant viral vectors for gene transfer? What is the risk associated with using packaging cell lines for vector production?
11. Provide two examples of how gene therapy is used to modulate the immune system to fight infection.
12. Describe one clinical trial for retrovirus-based gene therapy and adenovirus-based gene therapy and identify the most significant adverse effects that have been reported for each trial.
13. What can be incorporated into viral vector design to reduce genotoxic risk?
14. How can preexisting immunity to viral vectors be circumvented?
15. Name two advantages of adenovirus vectors as opposed to AAV vectors and vice versa.
16. How can adenoviruses be engineered to selectively replicate in tumor cells?

Answers

1. Embryonic stem cells are grown ex vivo after extraction of the inner cell mass from a blastocyst. Adult stem cells are found in vivo in many tissues, usually in the specialized environment of a stem cell niche that supports their asymmetric cell division.
2. Somatic cell nuclear transfer (SCNT) involves the injection of a donor genome into an enucleated egg, such that the embryo develops as a clone of the donor genome. This allows the generation of embryonic stem cells using the donor genome and, in principle, allows implantation into the uterus of a recipient female leading to pregnancy. There are ethical problems concerning the supply of fertilized human eggs and technical problems caused by incomplete reprogramming of the donor nucleus.
3. iPSCs are produced by transient expression of pluripotency genes in somatic cells, leading to reprogramming to form pluripotent cells resembling embryonic stem cells. The production of iPSCs allows pluripotent cells to be obtained from a patient without the need for SCNT. iPSCs can be used to derive differentiated cells for producing ATMPs for clinical application or disease modeling purposes.
4. In vivo gene therapy refers to the direct introduction of genetic material into the human body, whereas ex vivo gene therapy refers to the use of cells, which are genetically modified outside the body (i.e., ex vivo) prior to administration of these genetically modified cells into the human body. In the latter case, the genetic material is introduced into the human body using cells as "delivery system". See also "Gene Therapy" section.
5. Various cancers, autoimmune disorders, such as DM type I and Crohn's disease, neurological disorders, such as Parkinson's disease and Alzheimer's disease, myocardial infarction, and macular degeneration.
6. One of the concerns with stem cell-derived ATMPs is the possibility that rare pluripotent or multipotent cells in the product could give rise to tumors after administration to humans, i.e., tumorigenicity risk. Thus, the quality control of medicinal products is of paramount importance. Often, in particular, in the treatment of neurological diseases, it is not clear whether a progenitor, precursor or fully mature cell should be administered. Careful preclinical work is required in each clinical indication to establish the most effective approach. Where the strategy is designed to replace a cell that is lost in a particular disease, the environment into which the cell-based medicinal product is placed may not be supportive of cell survival and integration/persistence. In general, one needs to pay attention to providing a protective environment for the medicinal product
7. The first gene therapy clinical trial was initiated in 1990 for treating adenosine deaminase (ADA) deficiency. In this trial, patients with ADA deficiency were given peripheral blood lymphocytes treated with a retroviral vector expressing the ADA transgene.
8. Promoter is a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase and transcriptional factors. Enhancer is a short DNA sequence that can bind transcription factors or activators to enhance transcription levels of a gene from a distance. Insulators are genetic boundary elements that block the enhancer-promoter interaction or rarely act as a barrier against condensed chromatin proteins. Finally, operators and silencers are usually short DNA sequences close to the promoter with binding affinity to a set of proteins named repressors and inducers.
9. (a) Retrovirus trial

   Gene-directed enzyme-prodrug therapy. Cells transduced by the virus express an enzyme capable of converting a prodrug (in this case, ganciclovir) to a cytotoxic metabolite. This conversion cannot occur in cells that do not express the transgene, limiting the cytotoxic effect to transduced cells and their neighbors through the bystander effect.

Adenovirus trial

Correction of genetic mutations that contribute to a malignant phenotype. Cells transduced by the virus express a gene such as p53 that is necessary for controlled cell division and development. This prevents the uncontrolled growth and division associated with malignant disease.

(b) Retrovirus

Advantages—(i) Retroviruses can infect dividing cells which are the therapeutic target in this trial. Despite this fact, the transduction efficiency of this vector in vivo has been low. (ii) Retroviruses can induce long-term gene expression, which should be sufficient to effectively remove malignant tissue.

Disadvantages—(i) Retroviruses have the potential for inducing insertional mutagenesis in normal, healthy cells. (ii) Transgene expression is sometimes limited by the host immune response to cellular components acquired by the virus during large-scale production.

Adenovirus

Advantages—(i) Adenoviruses can infect dividing cells, the therapeutic target in this trial. (ii) Adenoviruses can induce high levels of transgene expression in short periods of time. (iii) Adenoviruses do not have the risk of insertional mutagenesis. (iv) It is relatively easy to produce large amounts of recombinant adenovirus sufficient for clinical use.

Disadvantages—(i) Transgene expression is transient, making readministration necessary for continued effect. (ii) Adenoviral vectors can induce a potent immune response. This limits the success of gene transfer after a second dose of virus and is associated with severe toxicity at certain doses. (iii) Preexisting immunity to adenovirus serotype 5 is common in the general population. This may also limit gene transfer.

(c) Drawbacks to gene-directed enzyme-prodrug therapy.

(i) Efficacy relies on efficient transgene expression and drug bioavailability. (ii) The therapeutic effect may spread to healthy cells through the bystander effect.

Drawbacks to gene correction therapy.

(ii) Gene correction may stop tumor growth but not eliminate it. (ii) Expression is not limited to malignant tissue.

(d) Other approaches for cancer gene therapy

(i) Immunotherapy. A vector expressing pro-inflammatory cytokines, co-stimulatory molecules, or tumor-specific antigens is injected directly into the tumor mass. This facilitates the formation of an antitumor immune response that targets and destroys malignant cells.

(ii) Virotherapy. A replication-competent virus naturally targeting cancers is directly injected into the tumor mass. The virus can induce cell death during replication in malignant tissue by producing cytotoxic proteins and subsequent cell lysis.

10. (i) The primary purpose of the packaging cell line is to provide genetic elements that support virus replication and assembly. These have been eliminated from the vector to prevent it from causing disease in the patient. (ii) The recombinant virus can incorporate elements for replication into its genome through homologous recombination during the production process. The potential for the generation of replication-competent virus (RCV) in this manner does exist for each vector but can vary due to specific features of a given packaging cell line.
11. (i) Gene transfer into autologous immunocytes to increase the immune system of a patient. (ii) Overexpression of protein inhibitors that interfere with virus infection and replication. (iii) Overexpression of known antigenic epitopes of the pathogen by DNA vaccination to stimulate an immune response.
12. (i) One trial employed aerosol administration of a recombinant adenovirus expressing cystic fibrosis transmembrane conductance regulator (CFTR) to treat cystic fibrosis (CF). Another trial employed a recombinant retrovirus expressing recombinant adenosine deaminase (ADA) to transduce autologous T lymphocytes isolated from patients for treating ADA deficiency-induced severe combined immunodeficiency (ADA-SCID). (ii) CF trial. Massive immune response to the recombinant viral vector.

    ADA-SCID trial. Lymphoproliferative leukemia is caused by insertional mutagenesis.
13. Self-inactivating configurations can be incorporated to enable the use of physiological and cell type specific promoters, which can reduce potential genotoxic risk. In addition, using viral vector systems with more random integration site profiles and with reduced ability to cause aberrant splicing.
14. Preexisting immunity to viral vectors can be circumvented by using serotypes that have no or low prevalence in the human population, including virus variants of nonhuman origin.
15. Adenovirus vectors can be used to achieve transient transgene expression, and it has a higher packaging capacity than AAV. On the other hand, AAV can be used to get potentially long-term transgene expression, and the risk for strong inducing (too) strong immune responses is lower.
16. Initially, these were generated by partial deletion of E1B, restricting genome replication to cells that lack p53 such as tumor cells. More recently, they have been

generated by removing a specific stretch of 24 amino acids from the E1A protein. These so-called AdΔ24 or AdΔRb vectors are unable to bind to the retinoblastoma (Rb) protein. Rb normally retains E2F, preventing the cells from entering the S-phase and thereby replicating the genome. Cancer cells often have an aberrated Rb pathway, facilitating the S-phase and AdΔRb24 replication independent from the Rb-binding activities of E1A.

References

Ankrum JA, Ong JF, Karp JM (2014) Mesenchymal stem cells: immune evasive, not immune privileged. Nat Biotechnol 32:252–260

Antoniou P, Miccio A, Brusson M (2021) Base and prime editing technologies for blood disorders. Front Genome Ed 3:618406

Anzalone AV, Randolph PB, Davis JR et al (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. https://doi.org/10.1038/s41586-019-1711-4

Armentano D, Sookdeo CC, Hehir KM et al (1995) Characterization of an adenovirus gene transfer vector containing an E4 deletion. Hum Gene Ther 6:1343–1353

Arrand JR, Roberts RJ (1979) The nucleotide sequences at the termini of adenovirus-2 DNA. J Mol Biol 128:577–594

Bajaj P, Schweller RM, Khademhosseini A et al (2014) 3D biofabrication strategies for tissue engineering and regenerative medicine. Annu Rev Biomed Eng 16:247–276

Bar and Benvenisty. EMBO J. 2019. https://doi.org/10.15252/embj.2018101033

Bartholomae CC, Arens A, Balaggan KS et al (2011) Lentiviral vector integration profiles differ in rodent postmitotic tissues. Mol Ther 19:703–710

Becker J, Fakhiri J, Grimm D (2022) Fantastic AAV gene therapy vectors and how to find them-random diversification, rational design and machine learning. Pathogens 11:756

Belete TM (2021) The Current Status of Gene Therapy for the Treatment of Cancer. Biologics 15:67–77

Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2:313–319

Biasco L, Ambrosi A, Pellin D et al (2011) Integration profile of retroviral vector in gene therapy treated patients is cell-specific according to gene expression and chromatin conformation of target cell. EMBO Mol Med 3:89–101

Biffi A, Bartolomae CC, Cesana D et al (2011) Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. Blood 117:5332–5339

Blaese RM, Culver KW, Miller AD et al (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science 270:475–80

Boorjian SA, Alemozaffar M, Konety BR et al (2021) Intravesical nadofaragene firadenovec gene therapy for BCG-unresponsive non-muscle-invasive bladder cancer: a single-arm, open-label, repeat-dose clinical trial. Lancet Oncol 22:107–117

Bots STF, Hoeben RC (2020) Non-human primate-derived adenoviruses for future use as oncolytic agents? Int J Mol Sci 21:4821

Brassard JA, Lutolf MP (2019) Engineering Stem Cell Self-organization to Build Better Organoids. Cell Stem Cell 24:860–876

Bravery CA (2015) Do human leukocyte antigen-typed cellular therapeutics based on induced pluripotent stem cells make commercial sense? Stem Cells Dev 24:1–10

Bravery CA, Carmen J, Fong T et al (2013) Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. Cytotherapy 15:9–19

Bukrinsky MI, Sharova N, Dempsey MP et al (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci U S A 89:6580–6584

Bulcha JT, Wang Y, Ma H et al (2021) Viral vector platforms within the gene therapy landscape. Signal Transduct Target Ther 6:53

Büning H (2013) Gene therapy enters the pharma market: the short story of a long journey. EMBO Mol Med 5:1–3

Byun S, Lee M, Kim M (2022) Gene therapy for Huntington's disease: The final strategy for a cure? J Mov Disord 15:15–20

Cadena-Herrera D, Esparza-De Lara JE, Ramírez-Ibañez ND et al (2015) Validation of three viable-cell counting methods: Manual, semi-automated, and automated. Biotechnol Rep (Amst) 7:9–16

Cattoglio C, Facchini G, Sartori D et al (2007) Hot spots of retroviral integration in human CD34 hematopoietic cells. Blood 110:1770–1778

Chand D, Mohr F, McMillan H et al (2021) Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. J Hepatol 74:560–566

Chen G, Abdeen AA, Wang Y et al (2019) A biodegradable nanocapsule delivers a Cas9 ribonucleoprotein complex for in vivo genome editing. Nat Nanotechnol. https://doi.org/10.1038/s41565-019-0539-2

Chen H, Xiang ZQ, Li Y et al (2010) Adenovirus-based vaccines: comparison of vectors from three species of adenoviridae. J Virol 84:10522–10532

Chen HH, Mack LM, Kelly R et al (1997) Persistence in muscle of an adenoviral vector that lacks all viral genes. Proc Natl Acad Sci U S A 94:1645–1650

Chiang C-Y, Ligunas GD, Chin W-C, Ni C-W (2020) Efficient nonviral stable transgenesis mediated by retroviral integrase. Mol Ther Methods Clin Dev 17:1061–1070

Chiesa R, Bernardo ME (2022) Haematopoietic stem cell gene therapy in inborn errors of metabolism. Br J Haematol 198:227–243

Chin MH, Pellegrini M, Plath K, Lowry WE (2010) Molecular analyses of human induced pluripotent stem cells and embryonic stem cells. Cell Stem Cell 7:263–269

Consentius C, Reinke P, Volk H-D (2015) Immunogenicity of allogeneic mesenchymal stromal cells: what has been seen in vitro and in vivo? Regen Med 10:305–315

Corradetti B, Gonzalez D, Mendes Pinto I, Conlan RS (2021) Editorial: Exosomes as therapeutic systems. Front Cell Dev Biol 9:714743

Croyle MA, Cheng X, Wilson JM (2001) Development of formulations that enhance the physical stability of viral vectors for human gene therapy. Gene Ther 8:1281–1291

Dai Y, Schwarz EM, Gu D et al (1995) Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. Proc Natl Acad Sci U S A 92:1401–1405

Daud AI, DeConti RC, Andrews S et al (2008) Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. J Clin Oncol 26:5896–5903

David RM, Doherty AT (2017) Viral vectors: The road to reducing genotoxicity. Toxicol Sci 155:315–325

Deichmann A, Brugman MH, Bartholomae CC et al (2011) Insertion sites in engrafted cells cluster within a limited repertoire of genomic areas after gammaretroviral vector gene therapy. Mol Ther 19:2031–2039

Deichmann A, Hacein-Bey-Abina S, Schmidt M et al (2007) Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. J Clin Invest 117:2225–2232

Dharan A, Bachmann N, Talley S et al (2020) Nuclear pore blockade reveals that HIV-1 completes reverse transcription and uncoating in the nucleus. Nat Microbiol 5:1088–1095

Edelstein ML, Abedi MR, Wixon J, Edelstein RM (2004) Gene therapy clinical trials worldwide 1989-2004-an overview. J Gene Med 6:597–602

Eichler F, Duncan C, Musolino PL et al (2017) Hematopoietic stem-cell gene therapy for cerebral adrenoleukodystrophy. N Engl J Med 377:1630–1638

Ertl HCJ (2005) Challenges of immune responses in gene replacement therapy. IDrugs 8:736–738

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156

Eyquem J, Mansilla-Soto J, Giavridis T, et al (2017) Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 543:113–117

Ferrua F, Aiuti A (2017) Twenty-Five Years of Gene Therapy for ADA-SCID: From Bubble Babies to an Approved Drug. Hum Gene Ther 28:972–981

Finn JD, Smith AR, Patel MC et al (2018) A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. Cell Rep 22:2227–2235

Fischer A, Hacein-Bey-Abina S (2020) Gene therapy for severe combined immunodeficiencies and beyond. J Exp Med 217. https://doi.org/10.1084/jem.20190607

Flotte T, Carter B, Conrad C et al (1996) A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. Hum Gene Ther 7:1145–1159

Frangoul H, Altshuler D, Cappellini MD et al (2021) CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. N Engl J Med 384:252–260

Fritz CC, Green MR (1996) HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. Curr Biol 6:848–854

Fumagalli F, Calbi V, Natali Sora MG et al (2022) Lentiviral haematopoietic stem-cell gene therapy for early-onset metachromatic leukodystrophy: long-term results from a non-randomised, open-label, phase 1/2 trial and expanded access. Lancet 399:372–383

Gaggar A, Shayakhmetov DM, Lieber A (2003) CD46 is a cellular receptor for group B adenoviruses. Nat Med 9:1408–1412

Galderisi U, Peluso G, Di Bernardo G (2022) Clinical Trials Based on Mesenchymal Stromal Cells are Exponentially Increasing: Where are We in Recent Years? Stem Cell Rev Rep 18:23–36

Gillmore JD, Gane E, Taubel J et al (2021) CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. N Engl J Med 385:493–502

Ginn SL, Amaya AK, Alexander IE et al (2018) Gene therapy clinical trials worldwide to 2017: An update. J Gene Med 20:e3015

Golchin A, Chatziparasidou A, Ranjbarvan P et al (2021) Embryonic Stem Cells in Clinical Trials: Current Overview of Developments and Challenges. Adv Exp Med Biol 1312:19–37

Gombold J, Peden K, Gavin D et al (2006a) Lot Release and Characterization Testing of Live-Virus-Based Vaccines and Gene Therapy Products, Part 1 Factors Influencing Assay Choices. Bioprocess Int 4:46–54

Gombold J, Peden K, Gavin D et al (2006b) Lot release and characterization testing of live-virus based vaccines and gene therapy products part 2: Case Studies and Discussion. Bioprocess Int 4:56–65

Goodwin J, Laslett AL, Rugg-Gunn PJ (2020) The application of cell surface markers to demarcate distinct human pluripotent states. Exp Cell Res 387:111749

Gupta RK, Abdul-Jawad S, McCoy LE et al (2019) HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. Nature 568:244–248

Gurumoorthy N, Nordin F, Tye GJ et al (2022) Non-Integrating Lentiviral Vectors in Clinical Applications: A Glance Through. Biomedicines 10. https://doi.org/10.3390/biomedicines10010107

Han DW, Tapia N, Hermann A et al (2012) Direct reprogramming of fibroblasts into neural stem cells by defined factors. Cell Stem Cell 10:465–472

Harrington K, Freeman DJ, Kelly B et al (2019) Optimizing oncolytic virotherapy in cancer treatment. Nat Rev Drug Discov 18:689–706

Herberts CA, Kwa MSG, Hermsen HPH (2011) Risk factors in the development of stem cell therapy. J Transl Med 9:29

Hybiak J, Jankowska K, Machaj F et al (2020) Reprogramming and transdifferentiation - two key processes for regenerative medicine. Eur J Pharmacol 882:173202

Ilic D, Devito L, Miere C, Codognotto S (2015) Human embryonic and induced pluripotent stem cells in clinical trials. Br Med Bull 116:19–27

Jia F, Wilson KD, Sun N et al (2010) A nonviral minicircle vector for deriving human iPS cells. Nat Methods 7:197–199

John Wiley and Sons LTD (2022) Gene Therapy Clinical Trials Worldwide. In: The Journal of Gene Medicine Clinical Trial Site. https://a873679.fmphost.com/fmi/webd/GTCT. Accessed 24 Dec 2022

Kaufman HL, Shalhout SZ, Iodice G (2022) Talimogene laherparepvec: Moving from first-in-class to best-in-class. Front Mol Biosci 9:834841

Kean TJ, Lin P, Caplan AI, Dennis JE (2013) MSCs: Delivery Routes and Engraftment, Cell-Targeting Strategies, and Immune Modulation. Stem Cells Int 2013:732742

Keller G (2005) Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 19:1129–1155

Kim JY, Nam Y, Rim YA, Ju JH (2022) Review of the Current Trends in Clinical Trials Involving Induced Pluripotent Stem Cells. Stem Cell Rev Rep 18:142–154

Kim W, Lee S, Kim HS et al (2018) Targeting mutant KRAS with CRISPR-Cas9 controls tumor growth. Genome Res 28:374–382

Kleinstiver BP, Pattanayak V, Prew MS et al (2016) High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529:490–495

Kohn LA, Kohn DB (2021) Gene Therapies for Primary Immune Deficiencies. Front Immunol 12:648951

Kurt IC, Zhou R, Iyer S et al (2021) CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. Nat Biotechnol 39:41–46

Kuzmin DA, Shutova MV, Johnston NR et al (2021) The clinical landscape for AAV gene therapies. Nat Rev Drug Discov 20:173–174

LaFountaine JS, Fathe K, Smyth HDC (2015) Delivery and therapeutic applications of gene editing technologies ZFNs, TALENs, and CRISPR/Cas9. Int J Pharm 494:180–194

Lander ES, Baylis F, Zhang F et al (2019) Adopt a moratorium on heritable genome editing. Nature Publishing Group UK. https://doi.org/10.1038/d41586-019-00726-5. Accessed 23 Dec 2022

Leibacher J, Henschler R (2016) Biodistribution, migration and homing of systemically applied mesenchymal stem/stromal cells. Stem Cell Res Ther 7:7

Lennon MJ, Rigney G, Raymont V, Sachdev P (2021) Genetic therapies for Alzheimer's disease: A scoping review. J Alzheimers Dis 84:491–504

Levenstein ME, Ludwig TE, Xu R-H et al (2006) Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells 24:568–574

Levine BL, Humeau LM, Boyer J et al (2006) Gene transfer in humans using a conditionally replicating lentiviral vector. Proc Natl Acad Sci U S A 103:17372–17377

Levine BL, Miskin J, Wonnacott K, Keir C (2017) Global Manufacturing of CAR T Cell Therapy. Mol Ther Methods Clin Dev 4:92–101

Li L, Allen C, Shivakumar R, Peshwa MV (2013) Large volume flow electroporation of mRNA: clinical scale process. Methods Mol Biol 969:127–138
Liang M (2018) Oncorine, the world first oncolytic virus medicine and its update in China. Curr Cancer Drug Targets 18:171–176
Liu C, Su C (2019) Design strategies and application progress of therapeutic exosomes. Theranostics 9:1015–1028
Liu J, Verma PJ (2015) Synthetic mRNA Reprogramming of Human Fibroblast Cells. Methods Mol Biol 1330:17–28
Lochmüller H, Petrof BJ, Pari G et al (1996) Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. Gene Ther 3:706–716
Louis N, Evelegh C, Graham FL (1997) Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology 233:423–429
Lui KO, Waldmann H, Fairchild PJ (2009) Embryonic stem cells: overcoming the immunological barriers to cell replacement therapy. Curr Stem Cell Res Ther 4:70–80
Macedo N, Miller DM, Haq R, Kaufman HL (2020) Clinical landscape of oncolytic virus research in 2020. J Immunother Cancer 8. https://doi.org/10.1136/jitc-2020-001486
MacGregor RR (2001) Clinical protocol. A phase 1 open-label clinical trial of the safety and tolerability of single escalating doses of autologous CD4 T cells transduced with VRX496 in HIV-positive subjects. Hum Gene Ther 12:2028–2029
Mahato RI, Rolland A, Tomlinson E (1997) Cationic lipid-based gene delivery systems: pharmaceutical perspectives. Pharm Res 14:853–859
Majhen D, Ambriovic-Ristov A (2006) Adenoviral vectors-how to use them in cancer gene therapy? Virus Res 119:121–133
Manilla P, Rebello T, Afable C et al (2005) Regulatory considerations for novel gene therapy products: a review of the process leading to the first clinical lentiviral vector. Hum Gene Ther 16:17–25
Manno CS, Pierce GF, Arruda VR, et al (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 12:342–347
Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638
Männistö M, Vanderkerken S, Toncheva V, et al (2002) Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. J Control Release 83:169–182
McKenzie DL, Collard WT, Rice KG (1999) Comparative gene transfer efficiency of low molecular weight polylysine DNA-condensing peptides. J Pept Res 54:311–318
Medina-Kauwe LK (2003) Endocytosis of adenovirus and adenovirus capsid proteins. Adv Drug Deliv Rev 55:1485–1496
Melo LG, Pachori AS, Gnecchi M, Dzau VJ (2005) Genetic therapies for cardiovascular diseases. Trends Mol Med 11:240–250
Merkert S, Martin U (2016) Targeted genome engineering using designer nucleases: State of the art and practical guidance for application in human pluripotent stem cells. Stem Cell Res 16:377–386
Miyoshi H, Takahashi M, Gage FH, Verma IM (1997) Stable and efficient gene transfer into the retina using an HIVbased lentiviral vector. Proc Natl Acad Sci U S A 94:10319–10323
Moiani A, Miccio A, Rizzi E et al (2013) Deletion of the LTR enhancer/promoter has no impact on the integration profile of MLV vectors in human hematopoietic progenitors. PLoS One 8:e55721
Morenweiser R (2005) Downstream processing of viral vectors and vaccines. Gene Ther 12(Suppl 1):S103–S110
Morgan RA, Walker R, Carter CS et al (2005) Preferential survival of CD4+ T lymphocytes engineered with anti-human immunodeficiency virus (HIV) genes in HIV-infected individuals. Hum Gene Ther 16:1065–1074
Mount NM, Ward SJ, Kefalas P, Hyllner J (2015) Cell-based therapy technology classifications and translational challenges. Philos Trans R Soc Lond B Biol Sci 370:20150017
Muul LM, Tuschong LM, Soenen SL, et al (2003) Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. Blood 101:2563–2569
Murry CE, Keller G (2008) Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661–680
Nemerow GR, Stewart PL (1999) Role of alpha(v) integrins in adenovirus cell entry and gene delivery. Microbiol Mol Biol Rev 63:725–734
Nguyen GN, Everett JK, Kafle S et al (2021) A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. Nat Biotechnol 39:47–55
Nilsson EC, Storm RJ, Bauer J et al (2011) The GD1a glycan is a cellular receptor for adenoviruses causing epidemic keratoconjunctivitis. Nat Med 17:105–109
Nori S, Okada Y, Nishimura S et al (2015) Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: oncogenic transformation with epithelial-mesenchymal transition. Stem Cell Reports 4:360–373
Panakanti R, Mahato RI (2009) Bipartite adenoviral vector encoding hHGF and hIL-1Ra for improved human islet transplantation. Pharm Res 26:587–596
Pearson S, Jia H, Kandachi K (2004) China approves first gene therapy. Nat Biotechnol 22:3–4
Pina S, Ribeiro VP, Marques CF et al (2019) Scaffolding Strategies for Tissue Engineering and Regenerative Medicine Applications. Materials 12. https://doi.org/10.3390/ma12111824
Platt RJ, Chen S, Zhou Y et al (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159:440–455
Pollock K, Stroemer P, Patel S et al (2006) A conditionally immortal clonal stem cell line from human cortical neuroepithelium for the treatment of ischemic stroke. Exp Neurol 199:143–155
Pupo A, Fernández A, Low SH et al (2022) AAV vectors: The Rubik's cube of human gene therapy. Mol Ther 30:3515–3541
Quintana-Bustamante O, Fañanas-Baquero S, Dessy-Rodriguez M et al (2022) Gene editing for inherited red blood cell diseases. Front Physiol 13:848261
Raes L, De Smedt SC, Raemdonck K, Braeckmans K (2021) Non-viral transfection technologies for next-generation therapeutic T cell engineering. Biotechnol Adv 49:107760
Ramakrishna S, Kwaku Dad A-B, Beloor J et al (2014) Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res 24:1020–1027
Ranga U, Woffendin C, Verma S et al (1998) Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. Proc Natl Acad Sci U S A 95:1201–1206
Raper SE, Chirmule N, Lee FS et al (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab 80:148–158
Reilly JP, Grise MA, Fortuin FD, et al (2005) Long-term (2-year) clinical events following transthoracic intramyocardial gene transfer of VEGF-2 in no-option patients. J Interv Cardiol 18:27–31
Rubin H (2011) The early history of tumor virology: Rous, RIF, and RAV. Proc Natl Acad Sci U S A 108:14389–14396
Sago CD, Lokugamage MP, Paunovska K et al (2018) High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing. Proc Natl Acad Sci U S A 115:E9944–E9952
Salmon F, Grosios K, Petry H (2014) Safety profile of recombinant adeno-associated viral vectors: focus on alipogene tiparvovec (Glybera®). Expert Rev Clin Pharmacol 7:53–65

Sandoval-Villegas N, Nurieva W, Amberger M, Ivics Z (2021) Contemporary Transposon Tools: A Review and Guide through Mechanisms and Applications of Sleeping Beauty, piggyBac and Tol2 for Genome Engineering. Int J Mol Sci 22. https://doi.org/10.3390/ijms22105084

Santos GW (1983) History of bone marrow transplantation. Clin Haematol 12:611–639

Sayed N, Liu C, Wu JC (2016) Translation of Human-Induced Pluripotent Stem Cells: From Clinical Trial in a Dish to Precision Medicine. J Am Coll Cardiol 67:2161–2176

Scadden DT (2006) The stem-cell niche as an entity of action. Nature 441:1075–1079

Schwartz SD, Regillo CD, Lam BL et al (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 385:509–516

Schwarzwaelder K, Howe SJ, Schmidt M et al (2007) Gammaretrovirus-mediated correction of SCID-X1 is associated with skewed vector integration site distribution in vivo. J Clin Invest 117:2241–2249

Selyutina A, Persaud M, Lee K et al (2020) Nuclear Import of the HIV-1 Core Precedes Reverse Transcription and Uncoating. Cell Rep 32:108201

Serva SN, Bernstein J, Thompson JA et al (2022) An update on advanced therapies for Parkinson's disease: From gene therapy to neuromodulation. Front Surg 9:863921

Shaw AR, Suzuki M (2019) Immunology of Adenoviral vectors in cancer therapy. Mol Ther Methods Clin Dev 15:418–429

Shimamura M, Nakagami H, Sanada F, Morishita R (2020) Progress of Gene Therapy in Cardiovascular Disease. Hypertension 76:1038–1044

Shinagawa M, Padmanabhan RV, Padmanabhan R (1980) The nucleotide sequence of the right-hand terminal SmaI-K fragment of adenovirus type 2 DNA. Gene 9:99–114

Shy BR, Vykunta VS, Ha A et al (2022) High-yield genome engineering in primary cells using a hybrid ssDNA repair template and small-molecule cocktails. Nat Biotechnol. https://doi.org/10.1038/s41587-022-01418-8

Singh S, Narang AS, Mahato RI (2011) Subcellular fate and off-target effects of siRNA, shRNA, and miRNA. Pharm Res 28:2996–3015

Smith BD, Grande DA (2015) The current state of scaffolds for musculoskeletal regenerative applications. Nat Rev Rheumatol 11:213–222

Staal FJT, Aiuti A, Cavazzana M (2019) Autologous Stem-Cell-Based Gene Therapy for Inherited Disorders: State of the Art and Perspectives. Front Pediatr 7:443

Stewart DJ, Hilton JD, Arnold JMO, et al (2006) Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) versus maximum medical treatment. Gene Ther 13:1503–1511

Stevanato L, Corteling RL, Stroemer P et al (2009) c-MycERTAM transgene silencing in a genetically modified human neural stem cell line implanted into MCAo rodent brain. BMC Neurosci 10:86

Stolberg SG (1999) The biotech death of Jesse Gelsinger. N Y Times Mag 136–140:149–150

Suerth JD, Labenski V, Schambach A (2014) Alpharetroviral vectors: from a cancer-causing agent to a useful tool for human gene therapy. Viruses 6:4811–4838

Surosky RT, Urabe M, Godwin SG et al (1997) Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. J Virol 71:7951–7959

Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Takebe T, Enomura M, Yoshizawa E et al (2015) Vascularized and Complex Organ Buds from Diverse Tissues via Mesenchymal Cell-Driven Condensation. Cell Stem Cell 16:556–565

Tchieu J, Kuoy E, Chin MH et al (2010) Female human iPSCs retain an inactive X chromosome. Cell Stem Cell 7:329–342

Thaker PH, Brady WE, Bradley WH et al (2015) Phase I study of intraperitoneal IL-12 plasmid formulated with PEG-PEI-cholesterol lipopolymer administered in combination with pegylated liposomal doxorubicin in recurrent or persistent epithelial ovarian, Fallopian tube, or primary peritoneal cancer patients: An NRG/GOG study. J Clin Orthod 33:5541–5541

Thier M, Wörsdörfer P, Lakes YB et al (2012) Direct conversion of fibroblasts into stably expandable neural stem cells. Cell Stem Cell 10:473–479

Thomson JA, Itskovitz-Eldor J, Shapiro SS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Trounson A, McDonald C (2015) Stem cell therapies in clinical trials: progress and challenges. Cell Stem Cell 17:11–22

Tucci F, Galimberti S, Naldini L et al (2022) A systematic review and meta-analysis of gene therapy with hematopoietic stem and progenitor cells for monogenic disorders. Nat Commun 13:1315

Uusi-Kerttula H, Hulin-Curtis S, Davies J, Parker AL (2015) Oncolytic Adenovirus: Strategies and insights for vector design and immuno-oncolytic applications. Viruses 7:6009–6042

Valente JFA, Queiroz JA, Sousa F (2018) p53 as the Focus of Gene Therapy: Past, Present and Future. Curr Drug Targets 19:1801–1817

Varderidou-Minasian S, Lorenowicz MJ (2020) Mesenchymal stromal/stem cell-derived extracellular vesicles in tissue repair: challenges and opportunities. Theranostics 10:5979–5997

Vasan S, Hurley A, Schlesinger SJ et al (2011) In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. PLoS One 6:e19252

Vestergaard HT, D'Apote L, Schneider CK, Herberts C (2013) The evolution of nonclinical regulatory science: advanced therapy medicinal products as a paradigm. Mol Ther 21:1644–1648

Verma IM (By Inder M. Verma on November 1 1990) Gene Therapy. Scientific American.

von Jonquieres G, Rae CD, Housley GD (2021) Emerging concepts in vector development for glial gene therapy: Implications for leukodystrophies. Front Cell Neurosci 15:661857

Walther J, Wilbie D, Tissingh VSJ et al (2022) Impact of Formulation Conditions on Lipid Nanoparticle Characteristics and Functional Delivery of CRISPR RNP for Gene Knock-Out and Correction. Pharmaceutics 14:213

Wang H, Li Z-Y, Liu Y et al (2011) Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and 14. Nat Med 17:96–104

Weber W, Fussenegger M (2006) Pharmacologic transgene control systems for gene therapy. J Gene Med 8:535–556

Wells DJ (2004) Gene therapy progress and prospects: electroporation and other physical methods. Gene Ther 11:1363–1369

Wiethoff CM, Nemerow GR (2015) Adenovirus membrane penetration: Tickling the tail of a sleeping dragon. Virology 479–480:591–599

Williams DA, Bledsoe JR, Duncan CN, et al (2022) Myelodysplastic syndromes after eli-cel gene therapy for cerebral adrenoleukodystrophy (CALD). In: MOLECULAR THERAPY. CELL PRESS 50 HAMPSHIRE ST, FLOOR 5, CAMBRIDGE, MA 02139 USA, pp 6–6

Wilmut I, Beaujean N, de Sousa PA et al (2002) Somatic cell nuclear transfer. Nature 419:583–586

Wright JF (2008) Manufacturing and characterizing AAV-based vectors for use in clinical studies. Gene Ther 15:840–848

Wright JF, Qu G, Tang C, Sommer JM (2003) Recombinant adeno-associated virus: formulation challenges and strategies for a gene therapy vector. Curr Opin Drug Discov Devel 6:174–178

Xia Y, Li X, Sun W (2020) Applications of Recombinant Adenovirus-p53 Gene Therapy for Cancers in the Clinic in China. Curr Gene Ther 20:127–141

Xin T, Cheng L, Zhou C et al (2022) In-Vivo Induced CAR-T Cell for the Potential Breakthrough to Overcome the Barriers of Current CAR-T Cell Therapy. Front Oncol 12:809754

Yan Z, Zhang Y, Duan D, Engelhardt JF (2000) Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. Proc Natl Acad Sci U S A 97:6716–6721

Yáñez-Mó M, Siljander PR-M, Andreu Z et al (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4:27066

Yang C-S, Li Z, Rana TM (2011) microRNAs modulate iPS cell generation. RNA 17:1451–1460

Ylä-Herttuala S (2019) Gene Therapy of Critical Limb Ischemia Enters Clinical Use. Mol. Ther. 27:2053

Yuan X, Wan H, Zhao X et al (2011) Brief report: combined chemical treatment enables Oct4-induced reprogramming from mouse embryonic fibroblasts. Stem Cells 29:549–553

Zaiss A-K, Liu Q, Bowen GP et al (2002) Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. J Virol 76:4580–4590

Zennou V, Petit C, Guetard D et al (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 101:173–185

Zhang W-W, Li L, Li D et al (2018) The first approved gene therapy product for cancer Ad-p53 (Gendicine): 12 years in the clinic. Hum Gene Ther 29:160–179

Zuris JA, Thompson DB, Shu Y et al (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol 33:73–80

Further Reading

BSI PAS 83:2012 (2012) Developing human cells for clinical applications in the European Union and the United States of America – guide. Publicly Available Specification PAS83:2012, The British Standards Institution, ISBN 978 0 580 71052 0

BSI PAS 84:2012 (2012) Cell therapy and regenerative medicine – glossary. Publicly Available Specification PAS84:2012, The British Standards Institution, ISBN 978 0 580 74904 9

BSI PAS 93:2011 (2011) Characterization of human cells for clinical applications – guide. Publicly Available Specification PAS93:2011, The British Standards Institution, ISBN 978 0 580 69850 7

Galli MC et al (2015) Regulatory aspects of gene therapy and cell therapy products : a global perspective. Springer, Cham [Switzerland]

Herzog RW, Popplewell L (2020) Gene therapy : a nefvw therapeutic direction? S. Karger, Basel

National Institutes of Health (2006) Regenerative medicine. National Institutes of Health, Bethesda, DC

Narang A, Mahato RI (2010) Targeted delivery of small and macromolecular drugs. CRC Press, Boca Raton, FL

Nóbrega C et al (2020) A handbook of gene and cell therapy. Springer, Cham

Schleef M et al (2001) Plasmids for therapy and vaccination. Wiley-VCH, New York, NY

Schleef M (2005) DNA pharmaceuticals: formulation and delivery in gene therapy. DNA vaccination and immunotherapy. John Wiley & Sons, Hoboken