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A Handbook of Gene and Cell Therapy

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Contents

Gene and Cell Therapy

1

The development of therapeutics to treat human disease has always been a major goal for biomedical research and innovation. Nevertheless, the complexity of the majority of human diseases poses an important and difficult obstacle to overcome. Moreover, the genetic contribution to these conditions complicates the targeting of endogenous and normal processes of cellular functioning, such as transcription and translation. In the past century, the understanding of DNA structure and the development of techniques to manipulate and recombine this molecule allowed the conception of new strategies that could use DNA as a therapeutic agent. In the 1970s, it was proposed for the first time that some human genetic conditions could be treated by the administration of exogenous DNA. The enormous technological advance in the field of biomedicine, with the human genome sequencing or the development of high-throughput techniques, for example, contributed to an effective application of gene therapy in the human context. Recently, the approval of several gene therapy medicines in Europe and the USA definitively established a new paradigm in human disease treatment and opened a new era for gene therapy.

1.1 The Concepts of Gene and Cell Therapy

As the name clearly implies, gene therapy refers to the use of genes as "drugs" to treat human diseases. In a simple way, **gene therapy** could be defined as a set of strategies modifying gene expression or correcting mutant/defective genes, which involves the administration nucleic acids - DNA or RNA - to cells. However, more elaborate and complete definitions for gene therapy can be found, especially the ones produced by regulatory agencies. According to the European Medicines Agency (EMA) and to the European Union (EU) directive 2001/83/EC, a gene therapy product consists on a biological medicinal product, which has the following characteristics [1]: (a) it contains an active substance, which includes 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; (b) its therapeutic, prophylactic, or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains or to the product of the genetic expression of this sequence. Gene therapy medicinal products do not include vaccines against infectious diseases. For the US Food and Drug Administration (FDA), gene therapy is the administration of genetic material to modify or manipulate the expression of a gene product or to alter the biological properties of living cells for therapeutic use [2].

If the idea seems like science fiction, the truth is that in 1972 Friedmann and Roblin discussed this possibility in a*Science* paper entitled: "Gene therapy for Human Genetic Disease?" [3]. In this very interesting and advanced paper for their time, the authors postulated that gene therapy

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could be used in the future to ameliorate genetic diseases. In a very simple, but very accurate image, they describe how a mammalian cell could be modified by an exogenous source of DNA, following a similar path to that used by a virus (Fig. 1.1). Despite predicting important advantages with this therapy, authors clearly opposed any attempt to perform gene therapy in humans in a foreseeable future. The authors provided three important reasons for that: (i) the understanding on gene regulation and genetic recombination was still inadequate; (ii) the relation between gene and disease phenotype was not clear for many genetic disorders; and (iii) there was no information on gene therapy side effects. Moreover, authors also raised some important ethical concerns about gene therapy, including eugenics, which are still important questions today in gene therapy applications.

Less than 20 years later in 1990, the first therapeutic clinical trial using gene therapy started in the USA at the National Institutes of Health (NIH) in Bethesda, Maryland. Michael Blease and French Anderson led the clinical trial in two patients with adenosine deaminase (ADA) deficiency, a monogenic condition causing severe immunodeficiency. In this trial, the two patients, Ashanthi DeSilva and Cindy Kisik, had their autologous T-cells treated *ex vivo* with the correct ADA gene (using retroviral vectors), which were then reinfused [4]. The success of this trial remains controversial as (i) the response of the patients to the treatment was modest and (ii) patients simultaneously received enzyme replacement therapy [5]. Nevertheless, it became the first clinical trial of gene therapy in history and provided an important boost and enthusiasm for the field.

However, in 1999 a major drawback led to the partial suspension of gene therapy clinical trials and to the reevaluation of others in the USA. In that year, Jesse Gelsinger died after a severe adverse immune reaction to a adenoviral vector used in gene therapy treatment, only 4 days after the procedure [6]. Gelsinger had ornithine transcarbamylase (OTC) deficiency, which is fatal for most of the carriers, although, in his case, it was partially controlled through drugs and a low-protein diet. Later, it was identified that there were several non-authorized alterations in the clinical protocol and not enough information in the informed consent [7].

Despite this important drawback, several clinical trials using gene therapy continued. One of those trials conducted in Europe treated 10 boys with X-linked severe combined immunodeficiency (X-SCID) [8]. However, after the gene therapy treatment, four boys developed leukemia and one died 60 months after the intervention. Posterior studies showed that the therapeutic transgene was inserted near an oncogene, leading to severe complications starting around

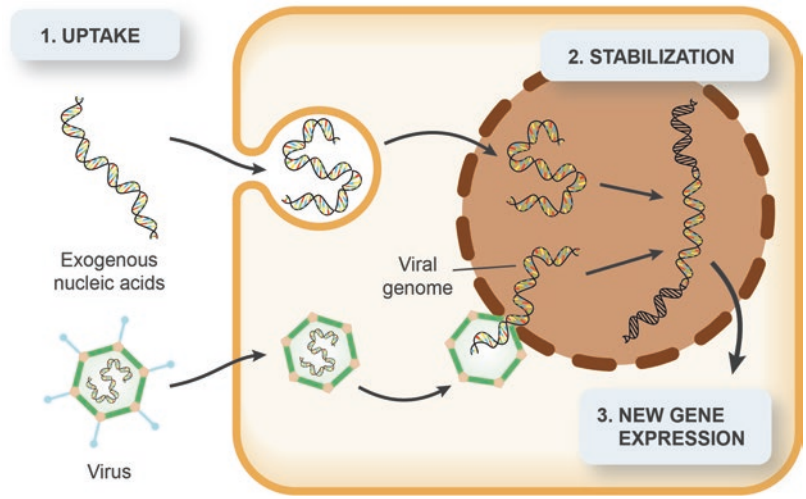

Fig. 1.1 Strategies for the genetic modification of a cell, as proposed by Friedmann and Roblin in 1972, in their *Science* paper entitled: "Gene therapy for Human Genetic Disease?". They posited that delivery of exogenous nucleic acids could be performed using different methods, by which exogenous genes could then be expressed in the modified cells.

30 months after the gene therapy. Nevertheless, in a 10-year follow-up of the intervention, the gene therapy was shown to have corrected the disease of the surviving participants [9]. In 2003, due to the adverse events in this study and several other concerns, the FDA suspended gene therapy clinical trials, arguing that not all the safety issues were addressed and more research was needed in the field [10]. Curiously, in the same year, China approved the first gene therapy product, Gendicine®, aiming to treat patients with tumors with p53 gene mutations [11]. However, the therapy was never approved in Europe, the USA, or Japan.

The first gene therapy medicine approved in these countries came in 2012, when Glybera® received marketing authorization in Europe for the treatment of lipoprotein lipase (LPL) deficiency, with the cost of 1.1 million euros, being, however, withdrawn from the market in the end of 2017 due to efficacy and low demand issues. Currently, several gene and cell therapy products are approved in Europe and the USA, including Strimvelis® to treat ADA-SCID, and more are in the pipeline for approval in the next years (see Sect. 1.11).

The concept and application of gene therapy is closely related to the idea of **cell therapy**, which can be roughly defined as an approach where cells are used as therapy or vehicle for therapy. Of course, cell therapy has been used for many years, considering, for example, blood transfusions and bone marrow transplants. Currently cell therapy per se has an enormous potential in regenerative medicine, even without genetic modifications of the cells. Nevertheless, the immunologic issues associated with cell transplants opened an opportunity for the combination of cell and gene therapy. In fact, several clinical trials have involved both gene and cell therapies, where defective (or not) cells are isolated from patients, treated with the therapeutic gene (using the appropriate vector), and then reinfused into the patient. Therefore, there is a clear overlap between both strategies Combined, they can be defined as a therapeutic intervention based on the administration of genetic material in order to modify or manipulate the expression of a gene product, altering the biological properties of living cells.

Despite all the drawbacks pointed before, research and the improvement in gene therapy knowledge and techniques continued. Important discoveries and advances like the RNA interference (RNAi) pathway, the Human Genome Project, or the production of induced pluripotent stem cells (iPSC) contributed to the continued interest in, and development of, gene therapy. More recently, the advance in gene editing techniques like TALENs or CRISPR provided a new boost in gene therapy, promising better, more accurate, and more effective forms to introduce or modifying genes.

For some, gene therapy was a promise that was never fulfilled, while others argue that the better is still to come. What offers no doubt is that gene therapy presents both advantages, like the possibility to effectively eradicate disease, and disadvantages, such as important ethical and safety issues that need to be addressed (Table 1.1). Currently, gene therapy is again in the spotlight of clinical and basic research, utilizing new techniques and taking advantage from the accumulated knowledge, the promising results of preclinical and clinical studies, and the interest of pharmaceutical companies due to the recent approval of several gene therapy products. Of

Table 1.1 Advantages and disadvantages of gene and cell therapy

Advantages	Disadvantages
Contributes to disease prevention	Modification of human abilities
Contributes to eradicate diseases	Changes the genetic pool
Helps to reduce the disease risk in future generations	Potential increase in diseases
Extends life expectancy	Safety problems
Avoids constant medication	High costs
Could replace defective cells	Ethical concerns
Unexplored potential	Short-time effect of some strategies
Allows a better understanding of how genes work	Might not be effective against complex diseases

course, this renovated interest makes gene therapy more prone to unethical procedures or poorly designed studies. Thus, important regulation procedures and careful attention to the studies are needed from the regulatory authorities, but also from scientists all over the world.

Designing a gene therapy study (in a preclinical or clinical context) is a complex process, where several variables must be considered ensuring the success and safety of the proposed therapy. Questions regarding the therapeutic target, the delivery system, or the immune response, for example, must be carefully studied and addressed before the application of the gene therapy. In the next sections, we will discuss some of the important issues that should be considered when designing a gene therapy study.

1.2 Types of Gene Therapy

The presence of genetic material in almost every cell in the human body makes these cells potential targets for gene therapy, including the germline cells. The major division between somatic and germline cells provides a categorization of gene therapy into two types, depending on the target cells. **Somatic gene therapy** refers to the interventions targeting the vast majority of human cells (somatic cells). On the other hand, we can speak of **germline gene therapy** if the targets of the intervention are the reproductive cells (Table 1.2). This very simple but clear classification advises that gene therapy directed to humans should be carried out exclusively in somatic cells. The germline gene therapy raises important ethical questions, being at least for now prohibited in Western countries [12]. Nevertheless, the advent of gene editing reopened the debate, and recently a panel of the US National Academy of Sciences considered the possibility of allowing embryo gene editing to prevent a disease, but only in rare circumstances and after further research [13].

Table 1.2 Main features of somatic gene therapy compared with germline gene therapy

Somatic gene therapy	Germline gene therapy
For the majority of the human cells	Changes will be transmitted to next generations
Alterations restricted to the patients	Unknown effect on future generations
Not passed on to future generations	Important bioethical issues
Less bioethical concerns	Technical difficulties in inserting genes in germ cells

Besides the important ethical and moral questions behind the gene editing of the germline, other technical questions also make it difficult: (i) currently the preimplantation diagnosis is able to identify and prevent several disease mutations, thus limiting the need for genome editing; (ii) the current procedures for zygote editing are not infallible, even in rodents; and (iii) offsite adverse and severe modifications can occur from the gene editing procedure. Despite the ethical and technical arguments, gene editing therapy was recently in the world spotlight, as in 2018 Chinese scientist He Jiankui claims that he performed gene editing in two human embryos, which were implanted and had already been born [14]. The scientific community and the world in general were astonished by this bold but highly questionable move, and the veracity of the claim is not completely assured.

For sure, the next few years will bring more debate and controversy on this matter, raising the need for rules and the maintenance of high ethical standards. Scientists and regulatory agencies are in the field, and the organization of world forums and summits on human gene editing will bring new regulation proposals. However, some controversies will certainly arise.

1.3 Gene Therapy Strategies

The application of gene therapy seems very straightforward if one thinks of genetic recessive disorders caused by a dysfunctional gene, where one normal copy of the gene could revert the disease phenotype and thus the only material to transfer is the correct gene (Fig. 1.2). This strategy, also called **gene augmentation therapy**, would be ideal to treat diseases caused by a gene mutation that leads to a malfunctioning or

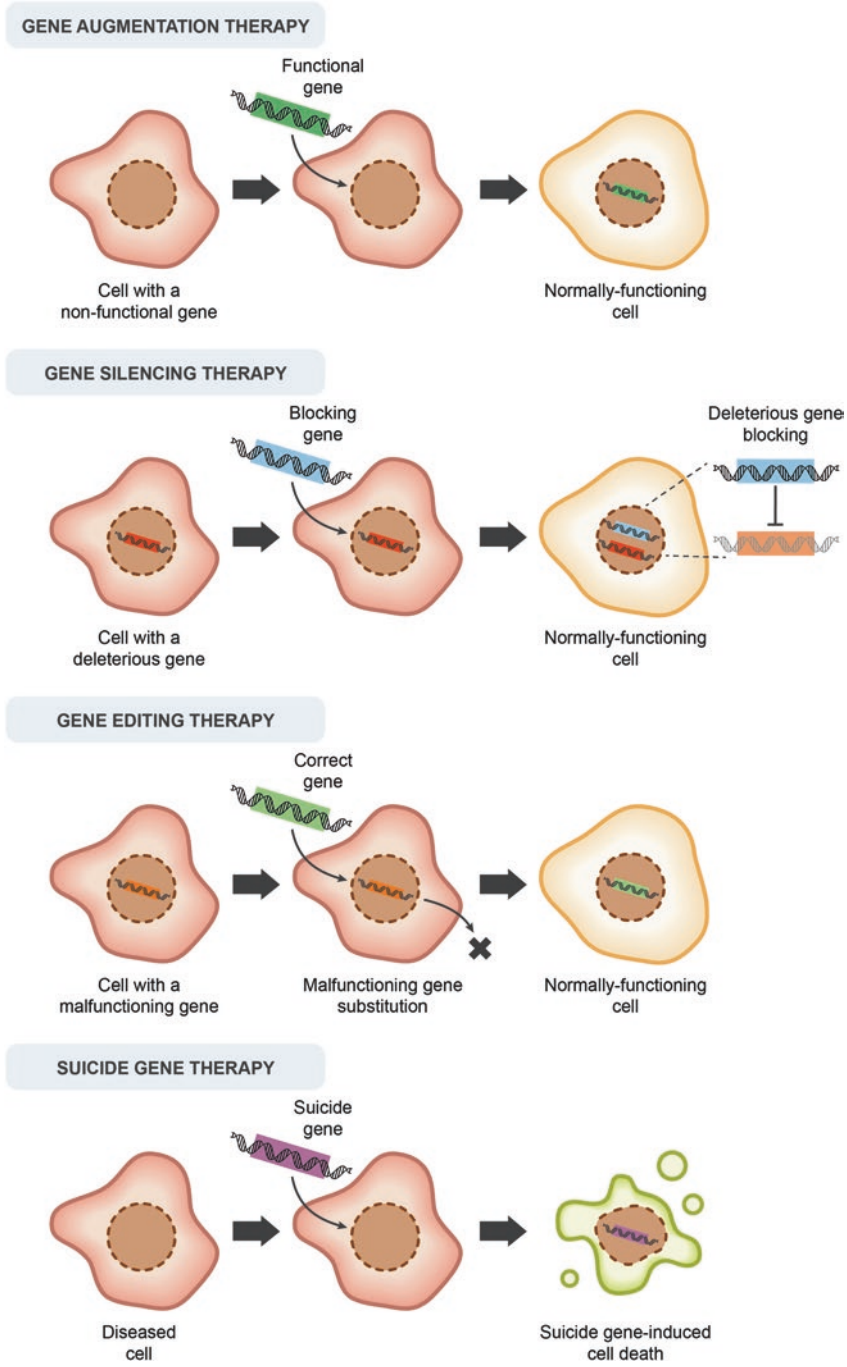

Fig. 1.2 Gene therapy strategies. In its simplest form, gene therapy could be performed by adding a new functional gene aiming to compensate a mutation or to improve cellular homeostasis – *gene augmentation therapy*. However, in some cases restoring a certain protein normal function may not be enough to mitigate the disease phenotype. For example, in genetic dominant diseases, mutant gene expression should instead be silenced, thus preventing the formation of the defective protein that causes the disease – *gene silencing therapy*. Recently, with the advent of gene editing tools, another strategy became available, in which a cell genome could be directly edited by removing a mutation or an entire gene and/or introducing a correct gene – *gene editing therapy*. All these three gene therapy strategies aim to revert cellular defects caused by malfunctioning genes. However, in certain diseases such as cancer, the aim is to cause cell death. In these cases, gene therapy could also be used, by introducing, for example, a toxic gene that will cause cellular apoptosis – *suicide gene therapy.*

deficiency of the resulting protein. In the treatment, adding a functional/normal version of the defective gene would theoretically guarantee the success of gene therapy. However, from a more practical point of view, this success is conditioned by at least two factors: (i) the levels of the normal protein produced by the inserted gene have to be sufficient and physiological, and (ii) the effects of the disease are still in a reversible state. This type of gene therapy was used in the first gene therapy clinical trial that was already mentioned for ADA-SCID, but it could also be effective for types of severe combined immunodeficiency or for cystic fibrosis (CF), among many others.

However, for many diseases, restoring the normal protein function would not be enough to revert the disease phenotype, and the expression of the mutant gene should actually be inhibited. This strategy, also named **gene silencing therapy** (or gene inhibition therapy) would be suitable, for example, for some genetic dominant diseases, some types of cancer or certain infectious diseases (Fig. 1.2). In the case of dominant diseases, the theoretical setup of this strategy would be to introduce a gene which could inhibit the expression of the mutant gene or that would interfere with the activity of the mutant protein. This approach became very feasible with the discovery of the RNAi pathway in 1998, by Andrew Fire and Craig Mello [15]. RNAi is an endogenous and conserved cellular pathway able to regulate gene expression through small RNA molecules that are complementary to mRNA (for more details, see Chap. 7). For gene therapy, the RNAi pathway offered an opportunity to use endogenous cellular machinery to control the expression of abnormal/defective genes. The gene silencing strategy has already been tested with success for several diseases in preclinical studies and currently is also being tested in clinical trials [16].

With the advent of **gene editing** techniques like TALENs or CRISPR, another strategy for gene therapy became available, aiming to edit the genome by removing a mutant gene and/or precisely correcting a gene (Fig. 1.2).

Of course, all strategies have problems and particularities that should be considered. For example, one of the main safety concerns with gene augmentation therapy is the possibility of the random insertion of the transgene, which could occur in problematic genome locations, such as the vicinity of oncogenes, tumor suppressor genes, or unstable genomic regions. On the other hand, the gene silencing or inhibition strategies, despite important successes, fail to completely shut down the expression of the target gene. Moreover, for gene silencing using the RNAi pathway, safety questions like off-target effects, long-term toxicity of the small RNA molecules or RNAi pathway saturation should also to be addressed and considered. Thus, the guided insertion of the transgene or the replacement of the abnormal/defective gene by a normal functioning gene appears as the ideal form of gene therapy, surpassing some of the problems presented by augmentation and silencing strategies of gene therapy. However, only recently have gene editing tools became easier to manipulate, allowing their use in the human gene therapy context. The enthusiasm in this field is so high that in 2016 a Chinese research group injected a person with cells edited by CRISPR-Cas9 [17]. Also in 2016, the first clinical trial using this system received a favorable opinion from an advisory committee at the US National Institutes of Health (NIH), aiming to be used in cancer therapy [18]. Nevertheless, potential off-target effects with these techniques or undesirable editing phenomena should also be considered and studied carefully.

The strategies mentioned above aim to restore the cellular homeostasis trying to revert the pathological abnormalities. However, in certain types of diseases such as cancer, the objective is to kill the defective cells. Gene therapy can also be used in this context, by using a transgene that (i) codifies for a highly toxic protein that kills the diseased cells or (ii) expresses a protein that marks the cell as a target for the immune system (Fig. 1.2). This type of gene therapy is sometimes called **suicide gene therapy** and is discussed in more detail in Chap. 9.

1.4 Choice of the Therapeutic Target

Another important issue in designing a gene therapy study is the choice of the target gene or cell, which entails a proper understanding of the genetic and molecular causes of a particular condition or disease. In the case of cell therapy, it is easy to conceive that the target will be the sick or defective cells. Nevertheless, it is crucial to consider important questions: (i) Do the cells used as therapeutics need to be treated with gene therapy? (ii) If stem cells are used, what would be the differentiation stage? (iii) What is the source of the cells?

In the case of gene therapy, the choice of the target is not so linear, as several options are available and their suitability depends on condition/disease pathogenesis. As mentioned before, it is easy to understand that, for a monogenic recessive disease, the gene therapy will consist in the addition of a "healthy" copy of the defective gene. However, in more complex pathologies, for example genetic dominant diseases, this strategy is not enough. In these diseases, one possible strategy for gene therapy would be the use of RNA and small RNA molecules to silence the expression of the abnormal causative gene. The different RNAi molecules, like siRNAs, shRNAs, and miRNAs, could be specifically designed to target the mRNA of the causative gene leading to

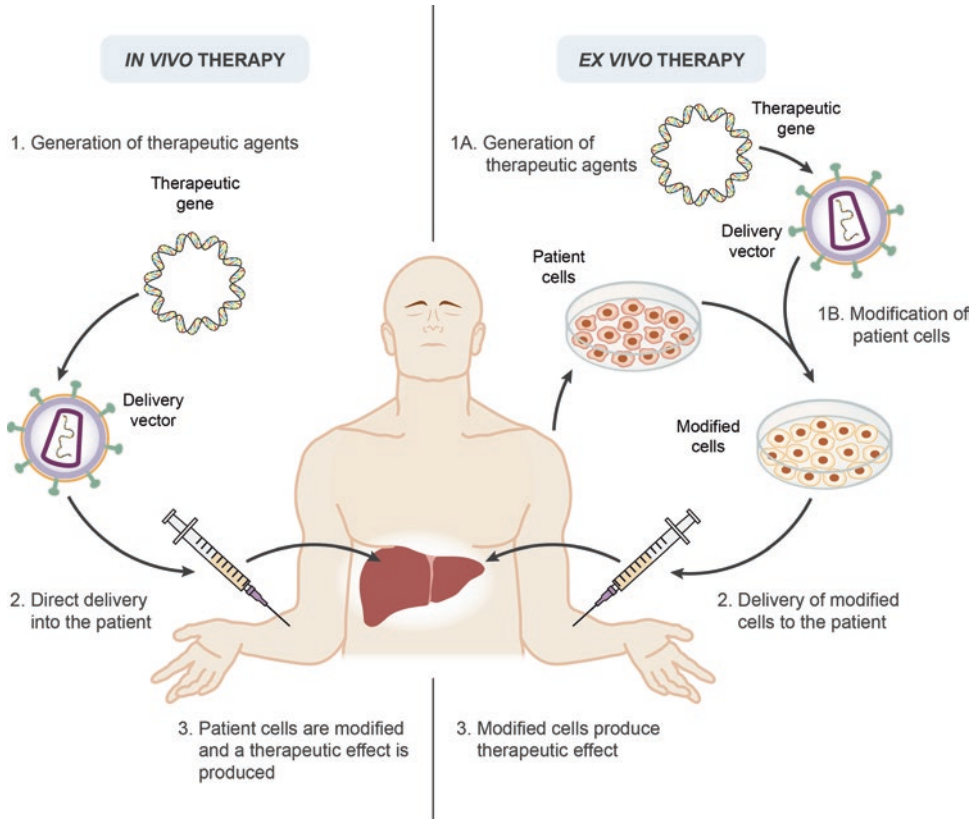

Fig. 1.3 Administration routes in gene therapy. An important consideration when designing a gene therapy application is to define the administration route to efficiently deliver the therapeutic gene to the target cells/organs. If the genes are directly delivered to the organism, the gene therapy is called *in vivo*. On the other hand, if the gene is delivered to cells outside the organism and then these manipulated cells are administrated to a subject, then the gene therapy is named *ex vivo*.

its cleavage or preventing its translation. Alternatively, a gene could also be used to treat dominant diseases aiming to improve the cellular function (like a gene to activate autophagy) or leading to cellular death (e.g., "suicide" gene therapy). Recently, the addition of a healthy copy of a gene also became an alternative for dominant diseases, if the abnormal copy is removed using gene editing tools.

Importantly, the disease pathophysiology should be carefully weighted when choosing a therapeutic target, as for many of the diseases affecting human health the use of cells would not be suitable.

1.5 Administration Routes

The localization of the target cells/organs is probably the main factor in deciding the administration route, along with the choice of the gene delivery vehicle, which is normally named **vector**.

Broadly, we can consider two options of administration routes for gene therapy: the direct delivery of the genes to organisms, also named *in vivo* therapy, and the delivery of genes to cells, which are then transplanted to the organism, named *ex vivo* therapy (Fig. 1.3). In the *in vivo* administration, the therapeutic sequence is delivered directly to the target cells, organs, or the whole body, which could be a less invasive method but is more prone to have off-target effects. On the other hand, in *ex vivo* therapy cells are treated outside the body and then transplanted to the patients, allowing more control of the treated cells, but being technically more complex (Table 1.3).

Nevertheless, this rather simple categorization of the administration routes is in fact far more complex. For example, in the direct *in vivo* administration, important questions should be considered: (i) Are the target cells/organs accessible to a direct application? (ii) In a whole organism administration, which fraction of the therapy reaches the target cells/organs? (iii) Could the remaining fraction be toxic? These and other questions need to be considered when designing a gene therapy study and before its application. For example, when targeting the central nervous system, the direct delivery route should consider the blood-brain barrier (BBB) and its selectivity. One way to circumvent the BBB would be the intraparenchymal injection into the brain or the infusion into the cerebrospinal fluid (the gene delivery to the central nervous system is discussed in detail in Chap. 4). However, these routes are highly invasive and greatly limit their selection in human patients. The *ex vivo* administration is also complicated by the source of the cells to be used. If allogenic cells are used, there is the problem of immune compatibility, whereas autologous cells sometimes are defective and are not suitable for the therapy.

Table 1.3 Comparison of *ex vivo* and *in vivo* administration routes used in gene therapy

In vivo (direct delivery)	*Ex vivo* (cell-based delivery)
Less invasive	More invasive
Technically more simple	Technically more complex
Vectors introduced directly	No vectors introduced directly
Safety check more difficult	Safety check easier
Reduced control of treated cells	More control of treated cells
Could be applied to a high number of diseases	Applied only to a small number of diseases
More definitive (depending on the delivery system)	Could be transient (cell lifetime)
Difficult to reach some cells/tissues	Possibility of accumulation of mutations
More off-target effects	Specificity for the treated cells

1.6 Delivery Systems

The delivery of exogenous genetic material into a cell or tissue is not a straightforward or easy process, as organisms developed several strategies and barriers to prevent it (see Chap. 4 for more details). Thus, one of the main issues to consider in a gene therapy strategy is the way to deliver the therapeutic sequence, that is, which delivery system is more suitable to ensure the success of the

therapy. In a broad way, two main groups of delivery systems are currently considered: the viral and the non-viral systems (Fig. 1.4). The **viral** systems take advantage from the broad diversity of viruses and their innate ability to infect/transduce cells. The key advantage of these systems is their high efficiency, whereas the main drawback is the safety concerns on using modified viruses. On the other hand, **non-viral** systems include several chemical or physical methods, which have as their principal advantage their safety profile, whereas the main disadvantage is their relatively low efficiency (Table 1.4).

The choice of the correct/ideal delivery system for a given gene therapy is dependent on several variables, including the size of the gene, the expected effect, and the toxicity profile, among others. The different delivery systems used in gene and cell therapy are described in more detail in Chaps. 2 and 3.

1.7 Expression and Persistence of the Therapy

Another important concern on gene and cell therapy applications is the expression levels of the inserted transgene/sequence, as it is virtually impossible to introduce a single copy of the transgene into the target cells. Importantly, the number of copies introduced is often different among the target cells. Both factors lead to (i) expression differences between target cells and (ii) increased expression levels relative to basal conditions. Moreover, if the transgene is integrated (using, e.g., retroviral vectors), expression will be continuous, producing expression levels that might be different from physiological basal levels (probably much higher), which could lead to toxicity effects. Thus, the implementation of a gene therapy in a clinical setting must ensure very tight and consistent regulation of transgene expression, which could be achieved using regulatable promoters. A proper gene regulation system should display several features, including [19]: (i) a low basal expression of the transgene, (ii) the expression should be triggered by the administration of a molecule and be responsive to a wide range of doses, (iii) be specific to the target cells/organs, (iv) do not interfere with endogenous gene expression, and (v) allow a rapid and effective induction or repression of the transgene expression.

Gene regulation systems can be categorized into two main groups: (i) **exogenously**-regulated systems, which use exogenous compounds to regulate gene expression and which are the most widely used in gene therapy applications, and (ii) **endogenously-**controlled systems, which rely on internal stimuli to control the transgene expression. Within the first group, the tetracycline (Tet) regulation systems are the most exploited and used tool for controlling gene expression, although others have been developed, like the rapamycin-regulated or the RU486-regulated systems. In the second group of systems, the promoter is sensitive to physiological parameters and conditions, such as glucose levels or hypoxia. However, this endogenous regulation is difficult, and thus most of the systems used are based on the administration of exogenous molecules.

Tetracyclines and their derivates like doxycycline (dox) have been widely used in the clinical setting as antibiotics, binding to the bacterial 30S ribosomal subunit and thus inhibiting protein translation. The **Tet systems** have two variants, the Tet-off system, which was the first one developed and that is based on the negative control by tetracycline [20], and the Tet-on system, that is currently more used and which is based on the positive control of expression by tetracycline [21] (Fig. 1.5). Both systems are based on the bacterial Tet operon, namely, in the Tet repressor protein (TetR) and the tet operator (tetO) DNA elements. In the eukaryotic **Tet-off system**, the TetR was modified with a transcription activation domain (AD) from the VP16 protein of the herpes simplex virus, creating a tetracycline-controlled transcriptional activator (tTA). Moreover, the tetO sequences were fused with a TATA box-containing eukaryotic promoter to construct the tetracycline-responsive promoter (P_{tet}). In the absence of tetracycline (or its derivates), the tTA will bind to the tetO sites in the P_{tet}, thus activating the expression of the downstream transgene. On the other hand, the pres-

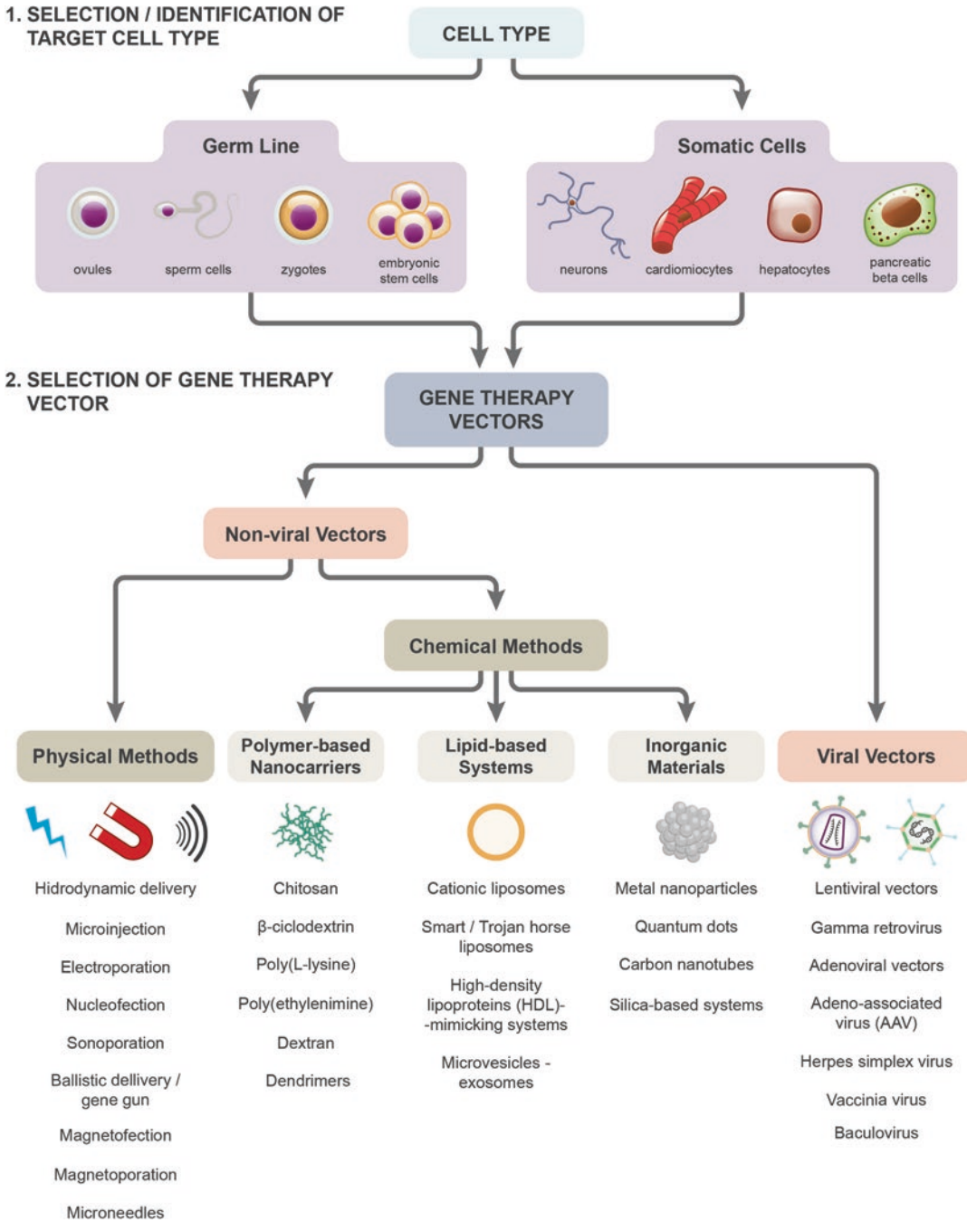

Fig. 1.4 Overview of the delivery systems used in gene therapy. Organisms and cells have developed several barriers to prevent the entry of exogenous genetic material. Therefore, overcoming these barriers to deliver the therapeutic gene is crucial to the success of gene therapy. In a broad manner, delivery systems for gene therapy can be classified into two groups: *non-viral vectors* and *viral vectors*. The first group refers to *physical and chemical methods*, such as microinjection or cationic liposomes. On the other hand, the second group is based on *engineered recombinant viruses* that are used to deliver the therapeutic transgene.