Development of adenoviral vectors armed with TNF-related therapeutic proteins for gene therapy

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Chapter 1

Introduction and thesis scope

The concept of gene therapy started brewing during the 1960s and early 1970s with the idea of using "good DNA" to replace the defective DNA in people who are affected by inherited diseases[1]. According to the American Society of Gene + Cell Therapy, gene therapy is defined as the introduction, removal, or change in the content of a person's genetic code with the goal of treating or curing a disease[2]." Simultaneously, the European Society of Gene & Cell Therapy (ESGCT) defines it as the "introduction of exogenous genes into cells with the goal of curing or improving a disease condition"[3].

Through the years (Fig. 1), gene therapy has been used to develop treatments for different diseases, e.g., monogenic disorders, cardiovascular diseases, infections, inflammatory diseases, and cancer[4]. The general course of action for gene therapy can go from (i) replacing or correcting defective genes; (ii) delivering a new or modified gene; to (iii) knocking down a gene or gene silencing[5,6].

Gene delivery

One of the essential parts of gene therapy is delivering the gene into the host cell; for this it is necessary to use vectors. The vectors are categorized into two main classes: the non-viral and the viral vectors[7].

Among the non-viral vectors are the lipid-based vectors (DOTMA), polymeric vectors (poly[L-Lysin], PLL; polyethylenimine [PEI]), inorganic nanoparticles, and the use of naked DNA[8]. These vectors have several advantages, like lower immunogenicity, and can carry larger genetic payloads; however, they can present lower transfection efficiency than viral vectors[8,9]. Recent examples of the use of this type of vector are the vaccines against the severe acute respiratory syndrome corona-virus 2 (SARS-CoV-2), lipid-based nanoparticles containing the RNA that encodes the full-length spike protein of the SARS-CoV-2: the mRNA-1273 SARS-CoV-2 vaccine from Moderna[10], and the BNT162b2 from Pfizer/BioNTech[11].

As its name implies, viral vectors use viruses as delivery vehicles taking advantage of their natural ability to infect cells. These viruses can be genetically engineered to harbor therapeutic genes while crippling or modifying their ability to replicate[12], such as the adenovirus vectors. The selection of the viral vector depends on the packaging capacity and integration into the genome; for

instance, retrovirus, lentivirus, and native adeno-associated viruses (AAV) can integrate into the host genome, while the adenovirus (Ad) and recombinant AAV (rAVV) cannot integrate[13].

In recent years, the adenovirus has been the most used vector in gene therapy clinical trials[4]. It has also been used in the development of vaccines against the SARS-CoV-2, such as the ChAdOx1 nCoV-19 (AstraZeneca), a replication-deficient chimpanzee adenoviral vector containing the spike protein from the SARS-CoV-2[14]; and the Ad26.COV2.S, a replication-deficient human adenovirus serotype 26 armed with the full-length of the stabilized SARS-CoV-2 spike protein (Johnson & Johnson)[15]

An integral part of the development of vectors for therapeutic use is their purification. Several chromatographic methods (e.g., size exclusion, metal chelate, and anion-exchange chromatography) have been used to facilitate the purification process, together with a concentration and buffer exchange step[16,17]. The advantages of using chromatographic methods are their rapidity, scalability, versatility, and capability to meet the adenoviral vector's requirements in the clinic[16].

Targeting cancer by gene therapy

Cancer is the second cause of death worldwide. The World Health Organization (WHO) defines it as "a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs[18]."

The search for more effective cancer treatments and fewer side effects leads to developing new approaches by using gene therapy. To date, 67.4% of the clinical trials on gene therapy are aimed at cancer treatment[19]; and, from 1998 to 2019, six cancer gene therapy products have been approved[20].

An attractive way to tackle cancer is to target its hallmarks, including continuous proliferative signaling, angiogenesis, and cell death evasion[21]. These hallmarks are present throughout different types of cancer, despite their origin.

Apoptosis or programmed cell death is a natural process that helps maintain the homeostatic cell balance; this mechanism can be altered or inhibited in cancer cells[22]. Therefore, triggering apoptosis by delivering apoptosis-inducing proteins

is the usual path to follow[23]. The most known apoptosis-inducing ligands are the Tumor necrosis factor-α (TNF-α), Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL). All of them belonged to the TNF super family[24].

From the TNF family, TRAIL and its receptors (DR4, DR5, DcR1, DcR2, and osteoprotegerin [OPG]) are primarily studied in cancer therapy because of TRAIL's ability to induce apoptosis in cancer cells while sparing the non-transformed ones [25]. Nonetheless, TRAIL has a short half-life in circulation, which makes a requirement for repeated administration. This characteristic is a disadvantage in the clinic, which can be solved with gene therapy[26].

RANKL (receptor activator of NF-κB ligand) is a homotrimeric transmembrane protein that can be cleaved and released as a soluble protein. RANKL binds to two receptors: the receptor activator of NF-κB (RANK), a type I homotrimeric transmembrane protein; and OPG, a soluble receptor[27]. The RANKL/RANK/OPG system was mainly known for its involvement in bone homeostasis and regulating immunity. However, recently has come to light the involvement of the RANKL/RANK/OPG axis in cancer cell migration[28], and it may also be associated with fibrosis[29]; this makes it an interesting subject to study. It must be remembered that TRAIL and RANKL have an affinity for OPG, a decoy receptor for both ligands.

Genome editing

Gene editing can be done by cleavage of a gene using the clustered regularly interspaced short palindromic repeats-CRISPR-Associated 9 (CRISPR-Cas9) technology[30], an RNA-guided system. The first member was identified in 1987 in *E. coli* [31]; since then, the presence of the CRISPR family has been reported in several prokaryote organisms[32], and several CRISPR-associated (*Cas*) genes have been identified[33]. For many years, CRISPR's purpose in bacteria was a mystery; nowadays, it is clear that CRISPR is involved in providing them immunity against foreign DNA (e.g., plasmids and viruses)[34,35] by cleaving the foreign DNA[36]. Thus, the CRISPR/Cas9 complex's ability to target DNA and induce double-strand cleavage makes it attractive for gene therapy. Furthermore, CRISPR technology can help to (i) screen the genome for "essential" genes that can be potential targets for cancer therapy[37]; (ii) develop cellular and animal models that can help to understand, diagnose and treat diverse diseases[38], such as cancer.

This thesis aimed to develop adenoviral vectors armed with therapeutic proteins to treat cancer or fibrosis.

First, in **chapter two,** we provide an overview of the studies done on the apoptosis-inducing ligands from the Tumor necrosis factor (TNF) superfamily and their receptors as cancer treatments. We discuss TNF-α, FasL, and TRAIL, which can induce apoptosis by binding to the death receptors (DR), and we provide strategies to overcome the ligands' natural limitations.

In **chapter three,** we show the production of adenoviral vectors and evaluate the use of anion-exchange chromatography along with ultrafiltration to purify five adenoviral vectors. Three adenoviral vectors are armed with a fusion protein containing TRAIL variants, and the other two carry RANKL variants. We developed a rapid and straightforward two-step purification process suitable for scale-up; furthermore, we obtained high-quality adenoviral vectors.

In **chapter four,** we use the CRISPR/Cas9 technology to knock down the epidermal growth factor receptor (EGFR) in a renal cell carcinoma cell line (RC21). The results show that the ablation of EGFR significantly limits tumor cell growth and activates the MAPK (pERK1/2) pathway. This study gave, as a result, an EGFR knockdown cell line, which was used in studies to evaluate the role of EGFR in the effectiveness of cancer treatments.

In **chapter five,** we assess the apoptotic activity of adenoviral-expressed scFv425-sTRAIL/DHER/4C7 proteins in cancer cells in **chapter three**. These fusion proteins contain a single-chain antibody fragment (scFv) that targets EGFR and TRAIL-specific proteins against the death receptors (DR4/DR5). The TRAIL fusion proteins show a higher effect in reducing cell viability than a combination of TRAIL protein with the anti-EGFR antibody.

In **chapter six**, we develop two RANKL adenoviral vectors, Ad.RANKL WT and Ad.RANKL_Q236D, to create a delivery system to target fibrotic tissue with adenoviral-expressed RANKL proteins without inducing bone remodeling. The activity of adenoviral-expressed RANKL WT and RANKL_Q236D was assessed and confirmed. RANKL_Q236D can avoid binding to exogenous OPG. Ad.RANKL Q236D is a powerful tool that might help for treating fibrosis.

Finally, in **chapter seven**, the findings of this thesis are summarized together with the future perspectives in the field of cancer and fibrosis gene therapy.

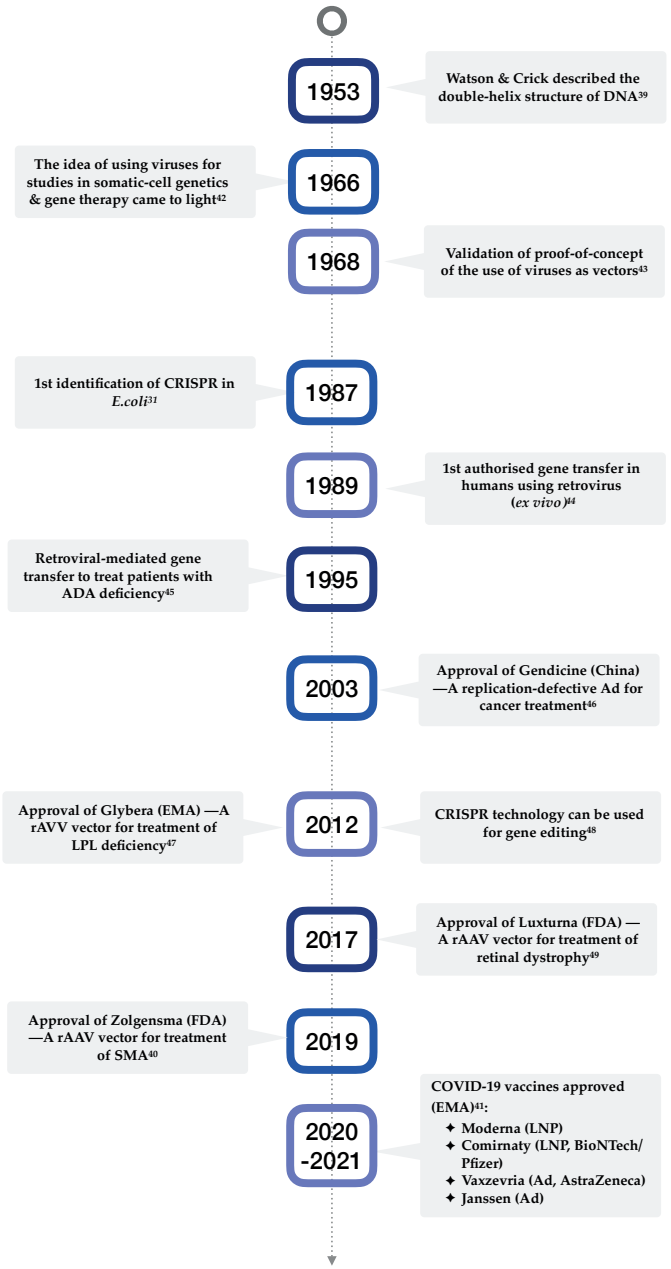

Figure 1. Timeline of the highlights in the field of gene therapy. **ADA,** adenosine deaminase deficiency; **LPL**, lipoprotein lipase deficiency; **SMA**; spinal muscular atrophy. References: [31,39–49].

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