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REVIEW

Viral gene therapy for breast cancer: progress and challenges

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ABSTRACT

Introduction: Breast cancer is the most common cancer in women all over the world. Furthermore, up to one third of breast tumors develop metastases that are resistant to standard therapies. Gene therapeutic strategies have been developed in order to specifically target cancer cells either directly or through the stimulation of antitumor immunity.

Areas covered: This review describes the therapeutic strategies that are currently under development to treat this disease using engineered viral vectors including: adenovirus, adeno-associated virus, lentivirus, poxvirus, reovirus, baculovirus, herpesvirus and oncolytic viruses. Advantages and disadvantages of these multiple gene therapy platforms are discussed in detail.

Expert opinion: Metastatic breast cancer is a perfect candidate for gene therapy approaches due to the presence of several tumor antigens and the aberrant expression of many molecular pathways. Oncolytic vectors are able to attack tumor cells while sparing normal cells and their activity is often enhanced by the administration of chemotherapy. However, more efforts are needed in order to reduce toxicity and to achieve better transduction efficiency. Improved preclinical models and a more critical patient selection for clinical trials, along with advances in gene therapy regulations, will surely facilitate the evolution of gene therapy for the treatment of metastatic breast cancer.

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1. Introduction

Gene therapy involves any procedure that modifies the patient's genetics in order to treat, cure, or prevent a medical condition or to improve their health condition. Whole genes, its associated regulatory elements, gene segments, or oligonucleotides may be delivered within the patient's target cells into the nucleus, so as to silence, stimulate, or regulate gene expression. Therapeutic genes, or transgenes, are delivered by mechanical devices or by gene vehicles called vectors. Gene vehicles can be viral (or bacterial), nonviral, or synthetic vectors, all of them having different levels of expression, immunogenicity, and biosafety (Table 1). When the genetic material is transferred directly into the cell, it is called 'transfection,' whereas the delivery of the transgene by a viral vector is called 'transduction.' These vehicles are one of the most important factors for the therapy to be effective, so they must be chosen with caution. Their interaction with the host immune system and their ability to penetrate the cell nucleus through all the barriers will determine how long the transgenes remain active within the host organism [1]. Delivery vehicles in viral gene therapy consist of viral vectors or engineered combinations with nonviral or synthetic vectors. Transgene transfer may be performed *in vivo* or *ex vivo*. In the first approach, the vector is injected directly into the patient's body, i.e. within the tumor or systemically. In the second one, cells of interest are collected from the patients and grown in culture with the corresponding gene vehicle, i.e. blood cells engineered to target tumor cells. Once genetically modified, these cells are reintroduced into the host [2]. Cancer research has achieved great advancements since the sequencing of the human genome in 2001 and the discovery of oncogenes and tumor suppressor genes [3,4]. However, there are no gene therapies approved yet by the US Food and Drug Administration (FDA) for the treatment of breast cancer, even though many targets are under investigation and already being evaluated in clinical trials. The targets may be cancer cells themselves, but also normal cells from the tumor microenvironment or the immune system. In this review, we will describe in detail the main viral vectors developed to date for the treatment of breast cancer and metastasis, some of which have reached clinical evaluation (Table 2). We will also give insight into future perspectives.

2. Viral vectors

2.1. Adenoviral vectors

Adenoviral vectors (Ads) are one of the most common viral vectors used in gene therapy due to their many advantages: (1)

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Article highlights

- Metastatic breast cancer is a good candidate for non-replicative and conditionally cytotoxic gene therapy approaches.
- Oncolytic vectors such as herpesvirus, adenovirus, reovirus, poxvirus and Newcastle disease virus are able to replicate within breast tumor cells and spare normal cells.
- Armed oncolytic vectors target additional pathways, such as angiogenesis and antitumor immunity
- Baculoviruses may be good carriers for breast cancer gene therapy.
- Viral gene therapy strategies are appropriate for *ex vivo* genetic modification of immune cells, such as DC vaccines and adoptive T cell therapy.
- The route of administration could dramatically affect the efficacy of gene therapeutic strategies.
- Neural and Mesenchymal Stem Cells may improve the biodistribution of gene therapy approaches for metastatic breast cancer.
- Viral systems are able to enhance the efficacy of chemotherapy.
- Further studies are needed to develop better gene carriers, improving targeting and transfection efficiency whereas reducing toxicity and avoiding rapid degradation.
- Appropriate tumor models may accelerate translation of experimental gene therapy approaches to the clinical practice.

This box summarizes key points contained in the article.

they are able to efficiently transduce dividing and non-dividing cells; (2) their viral genome is relatively easy to be modified by recombinant DNA technology; (3) they can be readily produced with high titers, 10^9 IFU/ml that can be concentrated to 10^{13} IFU/ml; and (4) the Ad genome remains episomal, which reduces the risk of insertional mutagenesis [7]. Ads are able to infect a wide variety of normal and cancer human cells, including cancer stem-like cells [8,9]. According to the ability of the Ads to replicate in target cells, recombinant Ads are classified as non-replicative or replicative, i.e. replication competent or oncolytic Ads. Non-replicative Ads carry a deletion in E1 and E3 regions, which makes the viruses unable to replicate in cells other than transgenic HEK293 Ad-producing cells, which bear Ad E1 genes [10]. One advantage of these Ads is their capacity to encode up to 7.5 kb of transgenic DNA [11]. Ad-mediated delivery of prodrug-activating enzymes, tumor suppressor genes, antiangiogenic factors, or immunomodulating genes can provide enhanced antitumor therapy with minimal toxicity to the host. Systemic therapeutic vaccination of breast tumor-bearing mice with a recombinant Ad expressing the full-length human epidermal growth factor receptor 2 (HER-2) inactivated for kinase function led to a robust polyclonal immune response to HER-2 and inhibition of tumor growth [12]. Intratumoral delivery of a non-replicative Ad encoding for Interleukin-12 (IL-12) in combination with an Ad that expresses the antiangiogenic peptide hormone angiotensin resulted in synergistic antitumor effect in murine models of breast cancer [13]. Antitumor efficacy has been demonstrated in preclinical models *in vivo* following local overexpression of proapoptotic molecules, including proapoptotic members of the Bcl-2 family, i.e. Bcl-xS and Bik [14,15].

Since metastases are resistant to traditional therapy, they are good candidates for experimental gene therapy strategies. It is well known that the intranasal route is the optimal route to achieve high transduction efficiency in the lung [16]. In fact, intranasal administration of gene therapy vectors has been shown to reduce the number and size of lung metastases [17,18]. Sustained transgene expression in the lung was achieved by intranasal delivery of an Ad-encoding angiostatin, which delayed the growth of lung metastases in a metastatic breast tumor model [18].

The main disadvantage of Ad-mediated gene therapy is that these vectors are highly immunogenic. In addition, virtually 100% of the global population possesses preexisting anti-Ad immunity, especially against the most common serotype (Ad5) used for the production of gene therapy vectors [19]. Clearance of the virus through neutralizing antiviral antibodies and/or cytotoxic T-cell-mediated immune responses could impair the efficacy of the therapy. Another important problem is the putative toxicity of systemic Ad administration. However, intratumoral administration is a good strategy to overcome this problem. Over the last few years, high-capacity adenoviral vectors (HC-Ads) have gained attention for sustained therapeutic transgene expression. HC-Ads lack almost all viral coding sequences, which makes them non-immunogenic and allow large packaging capacity (up to 35 kb) [20]. These vectors could be useful for therapeutic transgene delivery into breast tumors, as it has been shown in other tumors. Systemic administration of a HC-Ad encoding IL-12 [21] or a decoy receptor for Insulin-like Growth Factor (IGF-I) [22] led to prolonged transgene expression and inhibited the development of liver metastases in preclinical cancer models.

Limitations in the distribution of transgene expression using non-replicative Ads [23,24] motivated the development of oncolytic adenoviral vectors (OAVs), which selectively replicate in tumor cells leading to their lysis without affecting normal cells, as an alternative therapeutic strategy. Wild-type Ad E1A and E1B genes activate cell cycle by binding Rb or p53, which stimulates DNA synthesis in the host cell, increasing the availability of nucleotides for DNA replication. Conditional replication has been achieved by a deletion in the Ad E1A gene that impairs its binding to the Rb protein (Delta24), which limits viral replication in normal cells with functional Rb. However, in tumor cells with defective Rb function, the E1A gene is dispensable, and thus, OAVs can selectively replicate. Another strategy for tumor-specific replication is the expression of viral E1 genes under the control of tumor-specific promoters. The goal of OAVs in cancer therapy is to infect and lyse tumor cells *in situ*, but these Ads also have the potential to reach long-distance metastases. An additional advantage of this type of Ad is that tumor cell lysis allows the release of danger signals and tumor-associated antigens that can promote an additional immune-mediated antitumor effect [25]. Genetic manipulation allows specific viral replication in cancer cells [26], specific cellular tropism [27], and insertion of therapeutic transgenes for additional antitumor effect, such as immunostimulatory and antiangiogenic approaches in 'armed' oncolytic vectors [28]. The OAV, Ad.dcn encodes human decorin, which inhibits Transforming Growth Factor-β (TGF-β) signaling, tumor cell proliferation, and angiogenesis. Ad.dcn exhibited high replication rates and decorin transduction efficiency, yielding antimetastatic effects in a murine model of breast cancer bone metastases [29]. OAVs armed with immunostimulatory molecules increase the antitumor responses triggered by the virus. OAVs armed with IL-24 [30], CD40L [31], or Granulocyte Macrophage

Table 1. Advantages and disadvantages of viral gene therapy vectors.

Viral vectors	Advantages	Disadvantages
Adenovirus dsDNA Non-integrative	• Availability of non-replicative and oncolytic vectors • Efficient transduction in dividing and non-dividing cells • Ability to infect a wide variety of cells • Feasibility to modify its viral genome by recombinant DNA technology • High titers • Remains episomal • Cloning capacity up to 7.5 Kbp	• Highly immunogenic vectors • Preexisting anti-Ad immunity, especially Ad5 • Clearance of the virus through neutralizing antiviral antibodies and/or cytotoxic T-cell-mediated immune responses
Adeno-associated virus ssDNA Often non-integrative	• Replication dependent on a helper virus • Ability to establish latency and persist as episomes in the absence of a helper virus • Ability to infect dividing and non-dividing cells • Ability to transduce a broad range of tissues and to sustain long-term gene expression • Low immunogenicity and good safety profile	• In some rare cases, they can integrate into the host genome • Packaging capacity under 5 kb • Low transduction efficiency and specificity in certain cell types • They are less immunogenic than Ads, but antibody neutralization due to preexisting immunity against multiple Adeno-associated virus serotypes remains a common limitation
Lentivirus RNA Integrative or non-integrative	• Ability to infect both dividing and non-dividing cells • Efficient transduction of hematopoietic cells • Capacity for long-term stable expression • Latest generations exhibit increased biosafety • Modified LVs with non-integrative expression have lower risk of insertional mutagenesis • Cloning capacity up to 7.5 Kbp • Possibility of *ex vivo* approaches	• Integrative LVs entail risk of insertional mutagenesis • Titers up to 10^7–10^8 IFU/ml • Non-integrative LV expression is less stable because it remains episomal
Poxvirus dsDNA Integrative or non-integrative	• The enzymes required for transcription and replication inside the infected cell are provided by the viruses themselves • Normal cells are resistant to the infection and to the generation of viral progeny, whereas malignant cells are fully permissive. • Feasibility of genetic modification by homologous recombination • Cloning capacity up to 25 Kbp • Production of high-titer stocks • Transduction may be non-integrative or stable • Intravenous stability and efficient delivery to metastatic tumors • Good safety profile in humans	• Replication-competent viruses have increased risk of cross-infection • Possibility of causing undesired immunogenicity • The route of administration is only intradermal
Herpesvirus dsDNA Non-integrative Latency	• Large cloning capacity (up to 150 Kbp) • Episomal localization of its genome inside host cell • Availability of non-replicative and oncolytic vectors • Genetic manipulations confer the virus selectivity for cancer cells, while sparing normal cells	• Preexisting immunity • Its immunogenicity leads to transient transgene expression upon systemic administration • Low titers
Reovirus dsRNA	• Preferential replication in tumor cells • Radiation and chemotherapy act synergistically • Preclinical and clinical studies demonstrate antitumoral immune response • RV may be injected either inside the tumor or at a distant location, allowing systemic therapies • Possibility of *ex vivo* approaches	• Manipulation is not feasible • Unknown transgene-encoding capacity
Baculovirus dsDNA Non-integrative	• Ability to transduce different cell types from several species including human and murine cells • Efficient infection of a wide range of mammalian cells, including stem cells • Good safety profile, since neither integration of their DNA into the host cells genome nor viral replication have been reported • Preexisting immunity in humans has not been detected • Feasibility of manipulation • Capacity to encode up to 130 Kbp of transgenic DNA	• Baculovirus could be immunogenic upon systemic administration
Newcastle disease virus ssRNA Non-integrative	• Oncolytic vector • Good cell binding • Entrance into the cell via receptor-mediated endocytosis • Selective replication in tumor cells independently of cell proliferation • Absence of serious side effects	• Limited gene insertion • It does not allow the addition of tissue-specific promoters • Manipulation is not feasible

LV: lentiviral vector; BV: baculoviral vector.

Colony-Stimulating Factor (GM-CSF) genes led to robust anti-tumor responses in preclinical tumor models, even in combination with chemotherapeutic agents [32].

2.2. Adeno-associated viral vectors

Adeno-associated viruses (AAVs) are small replication-defective non-enveloped single-stranded DNA parvoviruses. AAVs can only replicate inside the cell in the presence of a helper virus, such as the adenovirus. However, AAV genomes can establish latency and persist as episomes in the absence of a helper virus or, in some rare cases, they can even integrate into the host genome, particularly in a specific region of the human chromosome 19 (AAVS1). AAVs are able to infect dividing and non-dividing cells [33]. These vectors seem to be attractive tools for therapeutic transgene

Table 2. Viral gene therapy clinical trials in breast cancer patients.

Immunotherapy	Approach	Action	Phase	Reference
Adenoviral vectors	Ad5CMV-p53 gene (I.T.) + chemotherapy	p53-mediated apoptosis	I	NCT00004038
			II	NCT00044993
	Ad. HSV-TK (I.T.)+ valacyclovir + radiotherapy	HSV-TK + valacyclovir-induced apoptosis of proliferating cells	II	NCT03004183
	Ad. HSV-TK (I.P.) + valacyclovir	HSV-TK + valacyclovir-induced apoptosis of proliferating cells	I	NCT01997190
	Ad.IL-12 (I.T.)	Immunostimulation	I	NCT00849459
	Ad.RTS.IL-12 (I.T.)	Immunostimulation	I/II	NCT02423902
	Irradiated tumor cell vaccine + Ad.Granulocyte macrophage colony stimulating factor	Immunostimulation	I	NCT00317603
			I/II	NCT00880464
	Ad. IFN-β (I.PI)	Immunogene therapy	I	NCT00066404
	Ad-HER-transduced DC vaccine (I.D.)	Therapeutic vaccination	I	NCT00197522
			I	NCT01730118
			I	NCT00162929
	Ad.ratHER-2 (I.D.)	Therapeutic vaccination	I	NCT00307229
Retroviral vectors	Retro-MDR1-peripheral blood progenitors + high-dose chemotherapy	Chemotherapy-resistant hematopoiesis	II	NCT00001493
	Anti-NY ESO-1 mTCR peripheral blood lymphocytes (I.V.)	T-cell-mediated lysis of NY-ESO-1-expressing tumor cells	II	NCT01967823
	Tumor-associated antigens (NY-ESO-1, MAGEA4, PRAME, survivin, and SSX)- Cytotoxic T lymphocytes(I.V.)	Cytotoxic T lymphocytes response against tumor-associated antigens-expressing tumor cells	I	NCT02239861
Oncolytic vectors	Replication-competent reovirus (Reolysin) + paclitaxel	Oncolysis of RAS-mutant cells	II	NCT01656538
	Replication-competent HSV-1 (HF10) (I.T.)	Preferential replication in tumor cells	I	NCT01017185
			I/II	NCT02779855
	Replication-competent poxvirus (JX-594) + cyclophosphamide (I.V.)	Preferential replication in tumor cells	I/II	NCT02630368
	Replication-competent HSV-1 (Talimogene laherparepvec) + paclitaxel	Preferential replication in tumor cells	I/II	NCT01017186
	NDV-modified irradiated tumor cell vaccine (ATV-NDV) (I.D.)	Preferential replication in tumor cells	II	Schirrmacher [5]
Poxviral vectors	PanVac vaccine + Granulocyte macrophage colony stimulating factor + docetaxel (I.V.)	Immunostimulation+ cytotoxicity	II	NCT00179309
	Recombinant vaccinia virus + MUC1 + IL2 (I.M.)	Immunostimulation	I/II	Scholl et al. [6]

I.T.: intratumoral; I.PI.: intrapleural; I.D.: intradermal; I.V.: intravenous; I.M.: intramuscular; NDV: Newcastle disease virus.

delivery since they are able to transduce a broad range of tissues and sustain long-term gene expression with low immunogenicity. These characteristics and their good safety profile [34] make them good candidates for antitumor gene therapy.

It has been shown that wild-type (wt) AAV type 2 infection induces apoptosis in different breast cancer cell lines and intratumoral injections of AAV2 into human MDA-MB-435 breast tumor xenografts in nude mice delays tumor growth and induces tumor necrosis [35]. Modified AAV2 expressing endostatine, an antiangiogenic compound, was able to inhibit tumor growth and to increase the survival of mice bearing mammary tumors when combined with paclitaxel [36]. Moreover, a novel hybrid vector recombining Ad and AAV (vAd–AAV) was developed to deliver a full-length antisense human telomerase RNA into MCF-7 human breast cancer cells, so as to suppress their telomerase activity. vAd–AAV achieved high gene transfer efficiency in mammalian cells and was able to integrate into the host's DNA. Results showed cytotoxicity in tumor cells and decreased proliferation [37].

Unfortunately, AAVs also present some disadvantages. Their packaging capacity is under 5 kb, their transduction efficiency and specificity in certain cell types still need to be improved, and, importantly, even though AAVs are less immunogenic than Ads, antibody neutralization due to previous exposure of the patient to multiple AAV serotypes remains a common limitation for successful gene therapy using these vectors [38,39]. Nevertheless, researchers have developed stronger and safer tumor-specific AAVs, which exhibit less undesired biodistribution. Targeted AAVs may be obtained from random phage display libraries, by screening AAV capsid peptides that mediate its binding to the target cell. These peptides are subsequently introduced into the AAV capsid region that is critical for receptor binding, therefore improving the specificity of cell tropism [40]. The main drawback of random AAV display peptide libraries is that they often retain collateral tropism, particularly towards the heart. This was overcome by engineering the AAV capsid with tumor-targeted peptides. In order to further reduce unspecific tropism, complementary target elements for heart-specific miRNAs were included, which abrogated transgene expression in the heart. This dual-targeted AAV vector was systemically administered to deliver a therapeutic suicide gene in an aggressive multifocal murine breast cancer model, suppressing tumor growth after one single injection, lacking the adverse effects of other targeted AAVs [38].

AAVs require the presence of a helper virus, such as Ads or herpesvirus (HSV), in order to enable replication and infection. Helper viruses allow complementary-strand synthesis before gene expression takes place. This is often a disadvantage because it limits the efficiency of AAV expression. Therefore, a self-complementary AAV (sc-AAV) has been developed, achieving higher transduction efficiency and faster gene expression. Its insert capacity is very low, no longer than 2150 bp, but researchers used it for packaging the short Pol III-based cassettes used for hairpin siRNA expression, which allows long-term expression of siRNA in targeted cells. Lastly, they assessed the

delivery of siRNA targeted to multidrug-resistance gene (MDR1), in multidrug-resistant human breast cancer cells (NCI/ADR-RES). P-glycoprotein multidrug transporter expression levels were significantly reduced, together with a long-term reversal of the drug-resistant phenotype of breast cancer cells [41].

AAVs have also been studied for the treatment of metastasis in different types of cancer. Antiangiogenic gene therapy approaches are a good strategy to kill metastatic tumor cells, since they exert sustained suppression of tumor blood supply. A recombinant AAV encoding mouse angiostatin was engineered for the treatment of a murine metastatic liver cancer model. This AAV was administrated via portal vein transfusion, improving the survival and exerting antimetastastic effects in murine models of lymphomas with liver metastases, without detectable toxicity [42]. Many similar antiangiogenic approaches have been evaluated with promising results [43–46], which suggests that similar strategies could prove useful for the treatment of metastatic breast cancer.

2.3. Lentiviral vectors

Retroviruses are RNA-enveloped viruses capable to reverse-transcribe their RNA into double-stranded DNA, which have the ability to integrate into the host genome and generate stable expression, even after the mitotic division of infected cells [47]. Retroviruses are divided into two classes: simple retroviruses as Moloney murine leukemia virus or complex lentiviruses like the human immunodeficiency viruses (HIV). While simple retroviruses can only infect actively dividing cells, lentiviruses are able to infect both dividing and non-dividing cells [48]. The ability of these vectors to easily enter into mammalian cells and their capacity for long-term stable expression makes them the perfect tool for gene therapy.

Lentiviral vectors (LVs) went through several modifications so as to increase biosafety and reduce pathogenic risk. Lentiviral genes are encoded in different plasmids in order to improve biosafety during viral production. First and second generation LVs are generated using three plasmids: (1) a packaging plasmid; (2) an envelope plasmid; and (3) the lentiviral transfer plasmid, which is replication incompetent and encodes for the insert of interest flanked by long terminal repeats (LTRs) that facilitate integration into the host genome. Second generation LVs are deleted of the accessory proteins Vpr, Vif, Vpu, and Nef, which are essential for HIV pathogeny but not for its replication. In these LVs, transgene expression is under the control of the weak promoter 5′LTR, unless an internal promoter is included in the expression cassette. Third generation LVs are generated using four plasmids, as the packaging system genes are encoded within two separate plasmids, which results in higher biosafety, but could yield lower LV titers. In third-generation LVs, the 5′LTR is partially deleted and fused to strong promoters, i.e. CMV or RSV for higher levels of transgene expression. An additional safety feature of these LVs is a deletion in the 3′LTR that renders it inactive. This sequence is transferred into 5′LTR after integration, leading to the inactivation of the promoter activity of both LTRs, and thus impairing transcription of the full-length virus after integration into the host genome [49]. Although all these features add safety to lentiviral production, the production of high-titer LVs remains difficult, typically yielding 10^7–10^8 IFU/ml. An additional safety feature consists in the mutation of the LV integrase, which impairs the integration into the host genome. Although this feature reduces the risk of insertional mutagenesis, non-integrative LV expression is less stable because it remains episomal and loses the transgenes after target cell replication, as it happens with Ads.

LVs transduce hematopoietic cells very efficiently, including antigen-presenting cells and lymphocytes, which makes them excellent tools for the *ex vivo* genetic engineering of immune cells for antitumor immunotherapeutic approaches. Systemic injections of these vectors may be useful for targeting distant metastases. LV transduction of dendritic cells (DCs) does not affect their immunophenotype, viability, or maturation capability [50]. LVs can be used to transfer tumor antigens [51] and pro-inflammatory genes [52] into DCs *ex vivo*, as well as to block the expression of tolerogenic molecules, such as IDO, IL-10, and TGF-β. Since LVs also efficiently transduce T lymphocytes, they are the vector of choice to genetically engineer autologous T cells *ex vivo* for cancer immunotherapy. LVs have been used to transduce peripheral blood T cells to express a chimeric antigen receptor (CAR), which consists of a tumor antigen-specific antibody fused to T-cell intracellular signaling domains. CAR–LV-transduced T cells were then reinjected into the host to specifically target antigen-expressing breast tumor cells, such as HER-2 and MUC-1 [53] or FR alpha [54].

Direct systemic injection of LVs encoding tumor antigens can also induce antitumor immunity. LVs efficiently transduce DCs *in vivo*, which in turn trigger an immune response against specific tumor antigens. In one study, a subcutaneous injection of a LV vaccine that encodes the extracellular domain of breast tumor antigen HER-2 led to antitumor immunity in BALB/c-HER-2/Neu transgenic mice bearing spontaneous breast tumors [55]. Moreover, immunization with LV vaccines encoding NY-ESO-1, a tumor antigen present in breast cancer and melanoma, led to potent antitumor cellular immunity without adverse effects in mouse cancer models [56].

LVs are also excellent vectors for transferring shRNA and microRNA (miRNA) into target cells. The blockade of hypoxia-inducible factor I (HIF-I), a transcription factor involved in the adaptation of tumor cells to hypoxic microenvironment, has been achieved using a LV encoding a shRNA specific for HIF-Iα. Infection of MDA-MB-231 cells with this LV successfully down-regulated HIF-1 expression and inhibited cell growth, migration, and invasion. Intratumoral injection of HIF-1α shRNA LV also elicited antitumor efficacy in mice bearing human MDA-MB-231 breast tumors [57]. LV-mediated delivery of shRNA specific for VEGF-C, which has been implicated in lymphangiogenesis and lymph node metastasis, was evaluated in MBA-MD-231 xenografts *in vivo*. Intratumoral injection of this vector exerted antitumor efficacy, inhibiting tumor growth and lymphangiogenesis and suppressing the development of lymph node metastases [58]. Another molecule of interest for breast cancer therapy is NRP-1, involved in the regulation of vascular endothelial cell migration, angiogenesis, tumor growth, invasion, and metastasis. It has been reported that inhibition of NRP-1 expression using NRP-1/shRNA LV inhibits breast tumor cell proliferation and

promotes apoptosis [59]. Considering that epithelial-mesenchymal transition (EMT) plays a crucial role in breast cancer tumorigenesis and metastasis, recent studies assessed the inhibitory effect of claudin1 (CLDN1), a tight junction protein, using a CLDN1/shRNA LV in human breast tumor cells. Suppression of CLDN1 inhibited proliferation, clonogenicity, and migration of breast cancer cells, downregulating the expression of the mesenchymal stem cells markers vimentin, Snai2, and SMA and upregulating E-cadherin, an epithelial cell marker [60]. Re-expression of a miRNA cluster, miR-200, using LVs in CLDN1 low tumors, also disrupted the EMT program, leading to reorganization of the tumor architecture, enhancing chemosensitivity, and decreasing the metastatic potential [61].

The versatility of LVs makes them perfect tools for immunogene therapy for the treatment of metastatic breast cancer. Although their production is complex and the titers obtained are not too high, their low immunogenicity and their ability to sustain long-term transgene expression are key features that make LVs first-choice viral vectors for many applications including cancer therapy.

2.4. Poxviral vectors

The *Poxviridae* family includes viruses that infect vertebrates and invertebrates. Based on their characteristics, poxviruses have been used in the development of human and veterinary vaccines as well as viral vectors for cancer therapy for the last three decades. Poxviruses are enveloped viruses with double-strand DNA genome of 130–350 kb encoding 300 or more genes [62]. The most remarkable feature of poxviruses is that, unlike other DNA viruses, they replicate in the cytoplasm of infected cells and the enzymes required for transcription and replication are provided by the viruses themselves [62]. Albeit cell receptors utilized by poxviruses have not been identified yet, it is believed that these viruses attach to ubiquitous cellular determinants due to their wide tropism for different cell types [62,63]. Inside the cytoplasm of infected cells, the virus replication cycle can proceed completely, generating viral progeny (oncolytic poxviruses), or abort due to incompatibility with the specific cell (non-productive infection) [62,63]. Since it has been observed that cells in active division are more permissive for poxvirus infection than quiescent cells [64], poxviruses have become excellent candidates for cancer therapy. However, up to date, the mechanisms responsible for oncolytic poxvirus selectivity for cancer cells are poorly understood [65]. It is believed that poxvirus selectivity for cancer cells is related to binding and entry steps; whereas normal cells are more resistant to the infection and to the generation of viral progeny, malignant cells are fully permissive. Thus, preferential amplification of poxvirus in the cancerous tissues can exert direct antitumor effect and stimulate effective antitumor immune responses that mediate regression of uninfected cancer cells [64].

The biological characteristics that make poxvirus promising candidates as vehicles for cancer therapy are (1) feasibility of genetic modification by homologous recombination and large transgene-encoding capacity, (2) simple production of high-titer stocks, (3) stability of virus preparations, (4) non-integrative transduction, (5) very large therapeutic index between cancer and normal cells, (6) intravenous (I.V.) stability and efficient delivery to metastatic tumors through blood vessels, (7) rapid spread within tumors, and (8) good safety in humans [66,67].

One of the first recombinant poxviral vectors used for oncolytic cancer therapy was the vaccinia virus (VV) with deleted viral thymidine kinase (TK) gene. Since TK is an essential enzyme for the pyrimidine synthesis pathway, *tk* gene deletion induces preferential replication in cells with high intracellular nucleotide pools as cancerous tissues. Thus, this vaccinia platform was employed to obtain recombinant vectors expressing various tumor antigens [68–74], cytokines, such as IL-2 and GM-CSF [75–78], and costimulatory molecules. An *in vivo* study evaluated the efficacy of a VV encoding for an antagonist of CXCR4, to block its interaction with chemokine CXCL12 in a triple-negative 4T1 breast carcinoma in syngeneic mice. CXCL4 is crucial for metastatic spread at CXCL12-expressing tissues. CXCL12 also stimulates tumor cell proliferation and angiogenesis and attracts immunosuppressive immune cells, such as Myeloid-derived suppressor cell (MDSCs) and regulatory T cells. This vaccine resulted in higher intratumoral concentration of the therapeutic protein than using the soluble antagonist CXCR4, with increased efficacy. More importantly, this treatment inhibited the development of spontaneous metastases after primary tumor resection and increased overall tumor-free survival [79]. An open-label phase I and II trial examined the safety and immunogenicity of a live recombinant VV encoding the tumor-associated antigen MUC1 and IL-2 in patients with advanced inoperable breast cancer recurrences. This trial demonstrated the absence of clinical adverse effects and environmental contamination by the vaccine. High doses could achieve strong anti-vaccinia antibody responses, and cytotoxic T cells were detected in some patients. The vaccine generated memory T cells (CD45RO) in tumor biopsies, corroborating the efficacy of the treatment for breast cancer patients [6]. Costimulation plays an important role in the immune response induced by cancer vaccines because tumor antigens are weakly immunogenic or functionally non-immunogenic [80]. Taking into account all these factors, a recombinant vaccinia denominated PANVAC-V was obtained and evaluated in patients with metastatic breast cancer. PANVAC-V encodes tumor-associated antigens mucin-1 (MUC-1) and carcinoembryonic antigen. PANVAC-V also encodes a triad of human T-cell costimulatory molecules designated TRICOM, composed of B7.1, intracellular adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen (LFA)-3 [81–84]. The first vaccination with PANVAC-V elicited a strong initial immune response, but its continuous use was limited by the generation of host-induced neutralizing antibodies against VV. In order to improve the performance of poxviral vaccines, non-replicative poxviruses such as fowlpox virus (F), canary poxvirus (ALVAC™), and modified vaccinia Ankara (MVA) have been engineered to encode the same epitopes than recombinant VV and used as boosts in vaccination schemes. This strategy minimizes the amount of viral protein to which the immune system is exposed. Thereby, vaccination with PANVAC-V/F has demonstrated both safety [85,86] and evidence of clinical activity in patients with metastatic breast cancer [80,87]. A phase II clinical trial evaluated PANVAC-V/F plus docetaxel (a chemotherapeutic drug) vs. docetaxel alone in patients with metastatic breast cancer,

showing a trend towards improved progression-free survival time in the combined treatment (NCT00179309). In conclusion, results from several studies and clinical trials suggest that poxviral vaccines constitute a promising tool as therapeutic vaccines against breast cancer.

2.5. Herpes viral vectors

HSVs are enveloped double-stranded DNA viruses with large cloning capacity that remain episomal after infection of target cells. HSVs can be used as non-replicative or replicative vectors, or amplicons, which lack most viral genes and allow cloning up to 150 kb. As it happens with Ads, AAVs, and Poxviruses, HSV vectors are immunogenic, which leads to transient transgene expression upon systemic administration. Preexisting immunity against HSVs also exist within the human population as HSV-1 and HSV-2 infect humans of all ages. HSV-1 is usually acquired orally and is highly infectious. One important quality of HSV-1 is that it can be used both as a delivery vector for cancer gene therapy and as backbone for oncolytic viruses [88,89]. The inherently direct cytotoxic effect of HSVs may be exploited for the development of viral-based oncolytic approaches. For mechanisms that remain largely unknown, human breast cancer cells are permissive to HSV and genetic manipulations confer the virus selectivity for these cells, while sparing normal cells [90,91]. Selective destruction of tumors leaves adjacent normal tissues undamaged, releasing progeny virions from the infected neoplastic cells once they are lysed by the viral replication.

HF10 is a spontaneously mutated oncolytic HSV-1 (oHSV). Conditional replication of HF10 in tumor cells was confirmed in patients with metastatic breast cancer by immunofluorescence assays of HF10-injected tumors [92]. HF10 has shown preclinical antitumor efficacy in many tumors, including breast cancer, and its effect can be enhanced by chemotherapy [93]. Administration of HF10 in immunocompetent mouse models of peritoneal disseminated colon carcinoma and breast cancer stimulated specific antitumor immune responses and provided resistance to rechallenge with malignant cells [94,95]. Promising preclinical data led to evaluation of this therapy in a pilot clinical trial of six patients with recurrent breast cancer that received intratumoral injection of the oncolytic virus HF10. Results showed efficient viral replication within malignant cells and the generation of antitumor immunity [96].

oHSV vectors may also be used against hypoxic cancer cells, which are usually refractory to conventional chemotherapy. A neurovirulence gene-deleted HSV strain has been recently developed to target breast cancer cells. Notably, hypoxic p53-deficient breast cancer cells can also be transduced by this vector, which makes it a good alternative for treating advanced breast tumors [97,98].

Armed oHSV has also been developed for improved antitumor efficacy. OncoVEX$^{GM\text{-}CSF}$ has been engineered to conditionally replicate in tumor cells and to express the immunostimulatory GM-CSF gene under the control of the strong and ubiquitous CMV promoter, which is the first armed oHSV tested in humans [99]. The antitumor effect of this vector seems to depend on the immune system, as in preclinical models, it eradicates injected and uninjected tumors and elicits immunological memory. Currently, this therapy (currently known as talimogene laherparepvec; T-VEC) is approved by the FDA for the treatment of melanoma skin tumors and is under investigation for breast cancer patients. A clinical trial is currently recruiting triple-negative breast cancer patients that will be injected intratumorally with the oHSV during chemotherapy with paclitaxel or prior surgery (NCT02779855).

2.6. Reoviral vectors

Reoviruses are double-stranded RNA non-enveloped icosahedral viruses with preferential replication in tumor cells. These oncolytic viruses mostly depend on activated Ras signaling, given that Ras-transformation promotes reovirus oncolysis [100]. The death of cancer cells is caused by the inhibition of the dsRNA-activated protein kinase [101]. Several studies show that reoviruses may act against cancer cells through innate and adaptive immune responses, which makes them attractive immunotherapeutic tools [100]. Reoviral vectors are currently being evaluated in clinical studies [102,103] with promising results in different types of cancer, such as breast cancer, prostate cancer, pancreatic cancer, malignant gliomas, advanced head and neck cancers, and metastatic ovarian cancers [104]. Reoviral oncolysis of tumor cells would mainly occur through apoptosis. Moreover, radiation and chemotherapy act synergistically in combination with reovirus antitumor immune responses [105]. Preclinical studies have demonstrated that reoviruses are able to achieve antitumor effects when injected either inside the tumor or at a distant location, encouraging the application of systemic therapies in breast cancer therapies. Of notice, reoviruses could also replicate in *ex vivo* approaches [106].

Reolysin®(Oncolytics Biotech® Inc.) is a wild-type unmodified type 3 Dearing strain reovirus that has proved to exert high anticancer activity. IND 213 is an open-label, randomized, non-blinded phase II trial that studies the therapeutic effect of intravenously administered Reolysin in combination with paclitaxel versus paclitaxel alone in patients with advanced or metastatic breast cancer (NCT01656538). Reolysin has also been tested in combination with docetaxel in a phase I trial for patients with advanced or metastatic solid tumor refractory to standard therapy, including breast cancer patients (REO 010). The combination was safe and well tolerated and achieved antitumor activity with complete responses. REO 009 was another open-label, dose-escalating phase I trial of Reolysin that evaluated a combined therapy with gemcitabine in patients with advanced or metastatic solid tumors, including breast tumors, achieving partial responses. In spite of the encouraging results of preclinical and clinical studies, further evaluations must be performed in order to improve reoviral antitumor immune responses.

2.7. Baculoviral vectors

Baculoviruses are enveloped DNA viruses that infect insects at larval stage. Their genomes are made up of circular double-stranded DNA ranging from 100 to 180 kb. Baculoviruses have proved to be useful biotechnological tools in the field of agriculture as well as for human and veterinary health. Over the last few years, baculoviruses have taken relevance in the

field of biomedicine, as they are able to transduce different cell types from several species including human and murine cells. Baculoviral vectors (BVs) are able to efficiently infect a wide range of mammalian cells, including stem cells, and they are very safe since neither integration of their DNA into the host cells genome nor viral replication have been reported. Preexisting immunity against BVs has not been detected in humans [107].

BVs have been extensively developed for the production of recombinant proteins and are now gaining attention for direct cancer therapy applications. BV-mediated transgene delivery has been studied in many different human cancer cells, including the human breast cancer cell line SkBr3, which overexpresses HER-2. BVs expressing human endostatin and angiostatin fusion protein exerted strong antiangiogenic and antitumor effects *in vivo* in murine cancer models [108]. BVs can also be used to transduce and activate DCs *ex vivo* for antitumor immunization. Infection of bone marrow-derived DCs with wild-type BVs induced upregulation of costimulatory molecules, Major histocompatibility complex (MHC), interferons, and other pro-inflammatory cytokines and results in improved antitumor immunity when injected in tumor-bearing mice [109]. Moreover, direct intradermal injection of wild-type BVs mixed with tumor cell lysates exerted antitumor efficacy in murine cancer models [110]. These observations indicate that although there is no reported preexisting anti-BV immunity, these vectors could be highly immunogenic upon systemic administration [111]. This immunostimulation could be useful when using BVs for immunotherapeutic applications, but can also lead to transient transgene expression when using BVs for gene delivery, which remains to be evaluated.

BVs can be readily manipulated using established and now commercially available technology, and they have a vast capacity to harbor exogenous DNA. The increasing number of specific molecular targets for breast cancer cells may prompt us to design individualized BV for treating patients with high efficacy in the near future.

2.8. Newcastle disease virus

Newcastle disease virus (NDV), also known as avian paramyxovirus type-1 (APMV1), belongs to the *Rubulavirus* genus of the family Paramyxoviridae in the order Mononegaviralis. It is an enveloped single-stranded RNA virus with a 16-kb genome, which has oncolytic activity in tumor cells, sparing normal human cells. NDV is under evaluation for its application in cancer virotherapy [112]. Although this virus leads to severe disease in birds, it only causes mild symptoms in humans [113]. Depending on the pathogeny of NDV in birds, this virus may be categorized as lentogenic (avirulent), mesogenic (intermediate), or velogenic (virulent). This classification is correlated to its oncolytic properties in human cancer cells. Velogenic and mesogenic strains are lytic in human cancer cells, whereas lentogenic strains are usually non-lytic. NDV proteins, such as hemagglutinin neuraminidase, are also implicated in the strains' virulence [114]. Some of the advantages of NDV as an anticancer agent include good cell binding, entrance into the cell via receptor-mediated endocytosis, selective replication in tumor cells independently of cell proliferation, and the absence of serious side effects [115].

NDV AF2240 is a velogenic strain that has been studied in several breast cancer cell lines, such as 4T1 [116] and MDA-MB-231 [113,117]. Researchers have shown that NDV AF2240 induces the apoptosis of MDA-MB-231 *in vitro* in a time-dependent manner, without causing toxicity in normal cells such as endothelial HUVEC cells and epithelial Hs578Bst breast cells [113,117].

A collaborative project funded by the National Cancer Council (MAKNA) was launched to develop NDV oncolytic vaccines using six different NDV strains, which were evaluated in several tumor cell lines such as MCF-7 and MDA-231, among others. AF2240, F and V4 strains induced significant oncolytic activity in these breast cancer cells, whereas Ijuk strain only showed apoptosis of MDA-231 cells. In most cases, oncolytic effects were observed only in cancer cells but not in normal cells. Notably, inactivation of NDV abrogated its oncolytic activity [118]. Of note, NDV AF2240 strain was also found to disseminate into the liver during intratumoral injection in murine breast tumor models [119].

One interesting study evaluated the efficacy of antitumor DC vaccines pulsed with viral oncolysates. Given that double-stranded RNA motifs are known to enhance maturation and activation of DCs, they can be loaded with NDV-infected tumor cells or oncolysates in order to achieve higher antitumor efficacy. In this study, DCs derived from breast cancer patients were pulsed with lysates from control or NDV-infected MCF-7 cells. DCs loaded with infected breast tumor cells successfully expressed costimulatory molecules and enhanced memory T-cell responses, in comparison with DCs pulsed by non-infected MCF-7. Furthermore, supernatants from DC–T cell cocultures showed higher levels of IFN-α, IFN-γ, and IL-15 when DCs were loaded with NDV-infected breast tumor cells [120].

A NDV-modified irradiated tumor cell vaccine was evaluated in three independent cohorts of patients with primary breast cancer, metastatic pretreated breast, or ovarian cancer. This study was performed to optimize this vaccine, providing relevant information for standardization and quality control [121]. Phase II clinical trials of these vaccination protocols revealed higher survival rates in the groups of patients that had received postoperative 'high-quality' vaccines versus 'low-quality' ones [5]. Taken together, these studies indicate that NDV is a versatile tool in virotherapy and immunogene therapy that could be further developed for the treatment of metastatic breast cancer.

2.9. Overcoming challenges in the biodistribution of viral vectors

2.9.1. Alternative routes of administration

The route of administration of viral vectors profoundly affects their biodistribution, which in turn can define their therapeutic efficacy and toxicity profile. For antitumor therapeutic therapies, viral vectors may be administered systemically or locally, either intratumorally or within the surrounding tissues. Viral vectors can be administrated by I.V., intraperitoneal, intracranial, intramuscular, intravascular (i.e. intraportal, intra-arterial,

and retrograde I.V.), intradermal, intratumoral, or subcutaneous injection and/or by inhalation, among others [122]. The oral route has not yet proved effective for the delivery of viral vectors because of the detrimental effect of gastric acids on the vector stability [123]. Each route of administration has several advantages and disadvantages according to dosage, specificity, complexity, efficiency, and feasibility. All these alternative routes of administration must be carefully studied *in vivo* in order to achieve optimal delivery and expression of the transgene with the least possible adverse effects.

Although in preclinical settings viral vectors are usually injected locally into the tumor, the systemic route of administration is often the route of choice to target distant metastases. However, the main drawbacks of systemic administration are the undesired biodistribution of viral vectors, which could lead to hepatotoxicity and multiple organ failure, as well as the neutralization of vectors due to the recognition of vector particles by the immune system, leading to a rapid clearance of the vectors. Ad vectors are scavenged by the reticuloendothelial system after systemic injection, especially by Kupffer cells (KC) in the liver. Vector clearance by KC is mediated by scavenger receptors, natural antibodies, and the complement, therefore contributing to an inflammatory response [124]. Ads have been engineered in order to improve the specificity of their tropism, i.e. Ad capsid modifications or tropism modulation through modified receptors. In addition, transient immunosuppression has been employed in order to inhibit anti-Ad immunity in hosts undergoing gene therapy strategies [125]. Researchers have shown that the addition of polyinosinic acid [poly(I)], a scavenger receptor A ligand, can prevent Ad sequestration by liver macrophages and KC, which would allow the administration of lower viral vector doses and therefore reduce liver toxicity, also improving transgene expression in target tissues [126]. Localized therapy to the lungs by intranasal route is a good alternative to treat pulmonary metastases without the putative risks of systemic administration [16]. A 5-week-long treatment consisting of intranasal administration of Ad–IL-12, twice a week, significantly blocked the development of lung metastases in murine models of cancer [17]. The development of lung metastases was also inhibited after intranasal administration of Ad-angiotensin in murine models of metastatic cancer [18]. Furthermore, intrapleural administration of Ads encoding cytotoxic (i.e. HSV-TK) or immunostimulating genes (i.e. IFN-β) reached clinical trial in breast cancer patients with lung metastases (NCT01997190; NCT00066404).

Although systemic administration of OAVs armed with TRAIL [127] or IL-24 [30] has shown to efficiently inhibit the growth of lung metastases in mice, optimal transduction efficiency can be achieved by the local injection of oncolytic viral vectors. OAVs may be applied in one area with great vascularization or they can even be distributed in multiple areas of the tumor, especially in the periphery of the tumor and in the border between healthy and malignant tissue [128]. An armed OAV, AdEHE2F, which encodes for two antiangiogenic factors, VEGFR inhibitor Fit-1A and an inhibitor of Notch signaling, elicited robust antitumor activity and disruption of tumor-associated angiogenesis after repeated intratumoral injection in a murine breast cancer model [129]. Bone metastases have also been targeted by I.V. injection of OAVs. Systemic administration of Ad.sTβRFc, an OAV armed with a decoy receptor for TGF-β, inhibited the growth of bone metastases and associated bone resorption in mice [130]. Nevertheless, local injection could improve efficacy and safety of OAV treatment for bone metastases. Intra-tibial injection of Ad5-Δ24-sOPG-Fc-RGD, an OAV armed with soluble osteoprotegerin, an inhibitor of bone resorption, limited the progression of bone metastases and inhibited osteoclast formation in a murine model of osteolytic bone metastases of breast cancer [131].

AAVs have been efficiently injected via several administration routes in different types of cancer for antiangiogenic approaches, such as intramuscular, intraportal, or even through the tail vein in mice [43–46]. However, the route of administration seems to profoundly shape the anti-AAV immune response. The injection of AAVs into the mouse tail vein triggers a CD4+ T-cell-dependent humoral response, while AAV delivery through the portal circulation leads to a T-cell-independent B-cell response. This is important because the concomitant administration of T-cell inhibitors is unable to improve therapeutic efficacy of AAVs after vector readministration [132]. The route of choice can also affect the extent of antitumor immunity induced by LVs. Administration of LV vaccine LV305 by subcutaneous, I.V., intramuscular, and intradermal injections in mice led to a cellular immune response with multifunctional CD8 T cells, capable of expressing more than one cytokine, except for the I.V. administration [56].

The use of cell-specific promoters allows targeting viral vectors to restricted cell populations following direct systemic administration. Transgene expression has been restricted to mature T cells using the CD4 gene promoter to control LV transgene expression [133]. On the other hand, LV transduction of human B cells has been efficiently achieved using the CD19 promoter [134] and the Ig kappa light chain promoter [135]. B cells have lately gained much attention in cancer therapy owing to their role in antitumor humoral immunity and immune tolerance. Targeting of specific immune cell populations can also be accomplished by modified LV vaccines, incorporating envelope glycoproteins from other viruses into the LV surface, a process named pseudotyping. LVs pseudotyped with measles virus glycoproteins that bind SLAM, a Signaling Lymphocytic Activation Molecule expressed on stimulated lymphocytes and DCs, restricts LV transduction to these immune cell populations after systemic administration [136]. Pseudotyping for major histocompatibility complex class II also improves LV targeting to DCs, reducing LV uptake by the liver *in vivo* [137]. These strategies could improve the efficacy of LV vaccines and decrease the toxicity of cancer treatments.

Despite the wide range of available administration routes, viral vectors often struggle with barriers during the transvascular transport. Although passive diffusion facilitates the exit of viral vectors from the vasculature into the tumor mass, tumor angiogenesis is usually irregular and may show aberrant tumor blood circulation and multiple sizes of vascular pores. This heterogeneity could be an obstacle for viral vector migration from the tumor blood vessels into the tumor microenvironment. Whereas the diameter of pores in the vessels of healthy tissues is about 2–6 nm, the size of tumor endothelial pores exhibits a wide range, from less than 1 nm to greater than 1 μm [138]. For instance, while

the vascular pores in primary brain tumors are bigger than 140 nm, the vascular pore size of brain metastases from breast cancer is approximately 10-fold smaller. Thus, large viral vectors would be unsuitable to treat these metastases, i.e. HSV virion diameter is close to 220 nm [138,139]. All these factors must be taken into account in translational and clinical studies.

2.9.2. Neural and mesenchymal stem cells as vehicles for viral vectors

In recent years, neural stem cells (NSC) or mesenchymal stem cells (MSC) have been proposed as useful tools to improve the biodistribution and efficacy of viral gene therapy approaches. These strategies involve the transduction of autologous NSC or MSC *ex vivo*, which are later injected into the host. Both NSCs and MSCs entail the capacity for self-renewal and, interestingly, the ability to migrate long distances, being specifically attracted by tumor cells [140,141]. These cells are thought to migrate towards tumor areas under the influence of chemoattractants, such as chemokines or growth factors, including stem cell factor, hepatocyte growth factor, VEGF, and c-kit that are secreted by proliferating tumor cells [142]. This tumor tropism can be exploited to target metastases using NSCs and MSCs transduced with viral vectors encoding anticancer agents. In a preclinical murine model of metastatic human breast cancer, NSCs were shown to preferentially target tumor metastases, regardless the target organ (i.e. lungs and lymph nodes or bone), rather than the primary tumor. This NSC tropism seems to be strongly influenced by the local production of IL-6, which is overexpressed in invasive breast cancer cells [143]. Another group revealed that human NSCs implanted into the brain of immunodeficient mice were able to migrate selectively into brain metastases of MDA-MB-435 human breast cancer cells, which were located in the opposite hemisphere [144].

Another advantage of NSCs and MSCs is that they are immunosuppressive cells, which would facilitate their escape from the immune system, providing long-term transgene expression from viral vectors that are normally immunogenic, such as Ads, poxviruses, and HSVs [140,141]. Researchers have developed human NSCs retrovirally transduced with transgenes expressing *Escherichia coli*-derived cytosine deaminase (CD) and human IFN-β as a treatment strategy for ductal breast cancer. CD converts the prodrug 5-fluorocytosine (5-FC) into its active chemotherapeutic form, 5-fluorouracil (5-FU), inhibiting DNA synthesis and finally inducing the death of MCF-7 and MDA-MB-231 human breast tumor cells, whereas IFN-β also kills them by apoptosis. Results showed selective migration towards the tumor cells and decreased tumor cell viability. Experiments *in vivo* confirmed antitumor efficacy in nude mice that received repeated local and intraperitoneal injections of NSC [145]. The therapeutic efficacy of MSCs transduced by oncolytic conditionally replicative adenovirus (CRAd) has already been evaluated in breast cancer models [146,147]. Researchers studied the therapeutic efficacy of MSCs loaded with E1A-mutant CRAd Adv-Stat3(-) which selectively replicates in breast cancer and melanoma cells, expressing high levels of anti-sense Stat3 complementary DNA, triggering apoptosis. *In vivo* experiments confirmed the tropism of MSCs towards the tumor after tail vein injection in nude mice, as well as the inhibition of tumor growth together with the increase of survival rates [146]. Moreover, systematically injected human MSCs loaded with CRAd into SCID mice bearing MDA-MB-321-derived pulmonary metastases achieved longer survival rates than I.V. injections of CRAd alone [147]. Although all these experimental approaches have accomplished promising results, MSCs and NSCs are usually studied in immunodeficient murine models of cancer, which may not mimic the tumor microenvironment in cancer patients. *In vivo* syngeneic cancer models are needed in order to evaluate the long-term expression of loaded MSCs and NSCs.

An additional potential disadvantage of this approach is that due to their capability of self-renewal and immunosuppressive characteristics, NSCs and MSCs could end up being oncogenic. Administration routes and different MSC sources may alter the effects on tumor growth [148–150], so researchers must pay special attention when working with these cells in antitumor treatments. Nevertheless, many alternatives have been developed to destroy remaining MSCs and NSCs, once the antitumor treatment is finished. In addition, transgene expression can also be interrupted in case of the development of adverse effects during gene therapy. A suicide system based on an inducible caspase-9 has been developed for controlling the growth and survival of MSCs after therapy [151]. Furthermore, researchers have engineered MSCs to express HSV-TK and s-TRAIL, allowing the killing of highly malignant glioblastoma cells followed by the elimination of the MSCs. This system induced caspase-mediated apoptosis of tumor cells and selective MSC sensitization to ganciclovir (GCV). Systemic administration GCV after the antitumor treatment effectively eliminated MSCs expressing HSV-TK [152].

3. Conclusion

Gene therapy has rapidly evolved over the last decades, and it continues to grow at an accelerated pace. Several viral systems have drawn attention for the treatment of breast cancer with impressive results; many of them are currently in the spotlight for clinical trials. Unfortunately, these systems still present limitations concerning biosafety regulations and toxicity, and they remain under investigation. Synthetic hybrids and combinations of these novel strategies with conventional therapies, such as chemotherapy and radiotherapy, appear to have synergic antitumor activity, even in multiple drug-resistant tumors. Summarizing, the utilization of viral systems for the delivery of nucleic acids as therapeutic agents seems an excellent strategy for the treatment of disseminated and metastatic breast cancer, but it is highly necessary to develop safer vectors.

4. Expert opinion

Since breast cancer is a global health problem that takes the lives of over 40,000 women per year in the U.S. only, there is

an urgent need of new strategies for treating patients with disseminated disease. The fact that breast tumors exhibit several associated antigens along with the aberrant expression of certain molecules and pathways makes breast cancer a perfect candidate for gene therapy. Nevertheless, gene therapy has not gained regulatory approval yet, due to the adverse effects caused primarily by former generations of viral vectors. Although all viral vectors exhibit drawbacks, i.e. the immune-mediated clearance of Ads, insertional mutagenesis, and low viral titers for LVs, researchers have tried to overcome them over the years. However, regulatory restrictions hinder the translation of newly engineered viral vectors or combination strategies to clinical trials, stretching the time between bench to bedside and delaying the availability of such treatments to cancer patients. In addition, once experimental gene therapy strategies reach clinical trials, their efficacy is often much lower than hitherto anticipated. This seems to rely heavily on the preclinical models used in translational oncology. The experimental metastases obtained by I.V. injection of tumor cells do not recapitulate the pathology of the natural metastatic process. This process cannot be mimicked by *in vitro* experimentation in cultures of metastatic cells either. Ideally, the preclinical evaluation of therapeutic strategies that target metastatic cells should involve multiple experimental models, i.e. short-term cultures derived from metastases obtained from cancer patients, and immunocompetent mice harboring chemically induced or inoculated primary tumors that metastasize spontaneously.

The use of rodent models can be a valuable tool to preliminary assess the feasibility of a novel antitumor strategy. However, many of the preclinical studies of gene therapy vectors, especially oncolytic viruses, have been performed in immunosuppressed mice. Considering that oAds and oHSVs are immunogenic, the absence of a fully functional immune system in these hosts may mask immune-mediated vector clearance and immunotoxicity caused by viral load. Thus, the usage of more stringent and relevant animal models in preclinical gene therapy may be crucial to reduce the distance between experimental development and clinical application. Larger animal models, such as canine and feline patients bearing spontaneous breast tumors and metastases, may constitute interesting preclinical models. Domestic animals bearing spontaneous tumors are ideal candidates to assess experimental viral gene therapy strategies that proved to be useful in rodent cancer models; their cancer histopathology and treatment are more similar to human patients, as well as their immunity against viral diseases. Larger animals also allow close monitoring and repeated sample collection by their owners, and their larger size allows better dose extrapolations and more feasible administration routes and schedules to treat primary and metastatic lesions. Both systemic and local administration of viral gene therapy vectors at the site of metastasis, i.e. intranasal, intrapleural, within the tumor mass, or into the tumor resection bed, could be better assessed and evaluated in larger animals. In addition, veterinary oncological patients could also benefit from these experimental treatments, which would actually become part of veterinary oncology clinical trials.

Patients' selection for clinical trials is also a limiting factor for gene therapy evolution. Enrolled patients are usually at very advanced stages of breast cancer, with several gene mutations and multiple drug resistance. Gene therapy would probably perform better antitumor effects during earlier stages of the disease, enhancing the efficacy of conventional treatments. Synergistic effects of gene therapy and chemotherapy may improve clinical outcomes, not only by enhancing antitumor efficacy, but also by decreasing the need of higher doses of the treatments and, therefore, their toxicity.

Ex vivo genetic modifications have opened the way not only to target malignant cells but also to modulate the tumor microenvironment. DCs and T lymphocytes, whether regulatory or cytotoxic, may be extracted from the host and then be grown in culture along with a therapeutic vector. Once transduced, they are ready to be injected back into the host so as to modulate antitumor immunity, often enhancing antigen presentation or downregulating the immunosuppressive tumor microenvironment.

Hybrids between oncolytic vectors and nonviral chemical methods for gene delivery, such as cationic lipid polymers, are a novel technology with a promising perspective for the future of gene therapy. These hybrids would exploit the advantages of both systems, circumventing their respective obstacles. However, the knowledge of this field is still poor, and it must be further developed for a better understanding. Although it is laborious and relatively expensive, viral gene therapy strategies exhibit so many advantages over traditional and recombinant protein-based therapies that they deserve the time and financial investment needed for their development and clinical application. The versatility of viral gene therapy strategies does not compare with any other oncological treatment. These vectors are able to upregulate or downregulate virtually any receptor, biomarker, or signaling pathway, whether nuclear, cytoplasmic, or membrane-bound, and in almost every type of cells, such as tumor, endothelial, or immune cells. Cell tropism can be modified by targeting the vector to specific cells or using cell-specific promoters. Spatial and temporal control of transgene expression can also be achieved by adding radio- or chemosensitive promoters. All these features make viral gene therapy vectors unique tools for personalized medicine in oncological patients, but research efforts must be doubled in order to reduce toxicity and achieve better transduction efficiency and, consequently, higher antitumor efficacy.

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