Progress in gene therapy for breast cancer and what comes next?

Bottai Giulia, MS,[1] Truffi Marta, PhD,[2] Corsi Fabio, MD,[2,3] Santarpia Libero, MD, PhD[1]

[1] Experimental Therapeutics Unit, IRCCS Clinical and Research Institute Humanitas, Via Manzoni 113 - 20089 Milan, Italy

[2] Department of Biomedical and Clinical Sciences "L. Sacco", University of Milan, via G. B. Grassi 74 - 20157 Milan, Italy

[*] Corresponding authors:

Libero Santarpia, MD, PhD - Experimental Therapeutics Unit, IRCCS Humanitas Clinical and Research Institute, Via Manzoni 56 - 20089 Milan, Italy, Phone: +39 02 8224 5173; Fax: +39 02 8224 5191, e-mail: libero.santarpia@humanitasresearch.it or liberosantarpia@yahoo.it

Fabio Corsi, MD - Department of Biomedical and Clinical Sciences "L. Sacco", University of Milan, Via G. B. Grassi, 74 - 20157 Milan, Italy, Phone: +39 0250319850-19858, e-mail: fabio.corsi@unimi.it

Abstract

Introduction: The possibility of correcting defective genes and modulating gene expression through gene therapy has emerged as a promising treatment strategy for breast cancer. Furthermore, the relevance of tumor immune microenvironment in supporting the oncogenic process has paved the way for novel immunomodulatory applications of gene therapy.

Areas covered: In this review, we describe the most relevant delivery systems, focusing on non-viral vectors, along with the description of the major approaches used to modify target cells, including gene transfer, RNA interference (RNAi), and epigenetic regulation. Furthermore, we highlight innovative therapeutic approaches, and the application of gene therapy in clinical trials for breast cancer.

Expert opinion: Gene therapy has the potential to impact clinical research. However, the safety and efficacy profile of delivery systems, and the specificity of gene modification must be improved. Further efforts are required to increase the clinical application of RNAi-based therapeutics, especially in combination with conventional treatments. Innovative strategies, including genome editing and stem cell-based systems, may certainly contribute to translate gene therapy into clinical practice. Importantly, immune-based strategies emerged as an attractive therapeutic opportunity for selected breast cancer patients. However, several technical challenges need to be addressed before using gene therapy as a concrete option for the treatment of breast cancer patients.

Keywords: breast cancer, gene therapy, nanovectors, immunotherapy, vaccine, clinical trials

1. Introduction

Breast cancer is the most frequently diagnosed cancer and a major cause of cancer death in women worldwide. Overall, breast cancer represents a complex and heterogeneous disease, encompassing several distinct entities with different molecular features and clinical behavior. Distinct genetic alterations and gene expression profiles differentially affect the development and progression of breast cancer subtypes, as well as individual patients' outcome and response to treatment. In this context, the possibility of correcting defective genes and modulating gene expression through gene therapy has emerged as a promising treatment strategy for breast cancer [1, 2]. The genetic modification of a target cells can be reached transferring genes, segments of genes, or oligonucleotides, including small-interfering RNAs (siRNAs) and microRNAs (miRNAs), by *in vivo* or ex vivo approaches [2]. Furthermore, the therapeutic potential of several genome editing techniques, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) systems, has been explored [2, 3]. Numerous strategies have been employed to transport the genetic materials to cancer cells, including viral and non-viral vectors [4, 5]. With the advancement of gene delivery techniques, several gene therapy clinical trials have been performed and are currently ongoing for cancer [2, 5-7]. However, the success of gene therapy can be limited by the heterogeneity between and within tumors, the inability to fully overcome the cellular and tissue barriers to deliver the therapeutic molecule, and the ineffectiveness to transform a sufficient number of cancer cells with the new genetic information. To enhance the therapeutic potential of gene therapy, combinatorial approaches with targeted- or chemotherapeutic- agents have been proposed [8, 9].

Besides genetic mutations and gene expression deregulation, emerging evidence suggests that the oncogenic process is consistently supported by the cancer microenvironment, especially by a defective tumor immune response [10]. These findings pave the way for novel application of gene

therapy, including the immunomodulation of cancer cells and the host immune system, and the delivery of factors that boost anti-tumor immunity.

2. Delivery technologies for human gene therapy

Historically, the fundamental limitation for *in vivo* clinical application of gene therapy is the lack of effective and safe delivery systems, which allow protection of the therapeutic molecules, efficient and specific uptake by the tumor, and ability of crossing biological barriers such as blood vessels and extracellular matrix. Several research and clinical studies have employed viral and non-viral vectors for the systemic delivery of the therapeutic molecules. However, both strategies present several limitations, and improvements of the efficiency and safety of delivery technologies are required prior to clinical application.

2.1 Virus-based approaches

To provide effective gene therapy, researchers have first evaluated strategies based on virus infection, which recapitulates a naturally occurring process of genetic intervention in mammalian cells. As a consequence, viral-based approaches were implemented as delivery carriers for nucleic acids in genetic disorders, and then as alternative treatment in non-Mendelian diseases such as cancer [6, 11, 12]. Viral vectors mainly rely on retrovirus, adenovirus, and adeno-associated viruses that were engineered to avoid viral replication and to replace viral DNA with the gene of interest. Therefore, host cells infection results in the introduction of exogenous genetic material, with transient or stable alteration of the genetic heritage of the cell [6, 11]. In particular, oncolytic viruses, such as herpes simplex virus type 1 (HSV-1), have been genetically engineered and explored for their anti-cancer therapeutic potential. They present cytotoxic effects coupled with a tropism for cancer cells, thus causing direct and specific destruction of malignant cells but also stimulating the host immune response [11, 12]. Despite clinical trials have shown relatively high transfection efficiency and beneficial results in a fraction of patients, the clinical use of viral vectors

has often been severely limited by intrinsic disadvantages, including insertional mutagenesis, small cargo capacity, inability to reach inaccessible tumors due to loco-regional administration [12, 13]. Importantly, virus-based approaches raised some safety issues, leading to reconsideration of the clinical application of viral vectors, thus accelerating the development of alternative non-viral carriers.

2.2 Non-viral nanovectors

Facing the huge potential of gene therapy in cancer, non-viral systems have been studied and developed to optimize DNA and RNA transfection of cancer cells. Non-viral strategies exploited for gene delivery include transduction of naked nucleic acids favored by physical intervention on cell permeability or provided through the aid of chemical compounds [2, 4, 14]. In particular, nanotechnology has revealed great potential in both increasing efficacy and reducing toxicity of chemotherapy in preclinical and clinical studies of breast cancer [15, 16]. Furthermore, nanovectors have been explored for breast cancer gene therapy, including RNA interference (RNAi) approaches, suicide genes delivery, and generation of nano-platforms for the co-delivery of chemotherapeutics and RNA molecules [17-20]. A schematic overview of non-viral systems, different cargos and functionalization strategies is shown in Figure 1.

Advances in using nanoparticles as gene delivery vectors are based on the possibility to develop various types of carriers for improving the pharmacokinetic and pharmacodynamic profiles of oligonucleotides, which usually display short half-life and rapid clearance, overcoming undesirable toxicity and immunogenicity, and enabling cellular membrane crossing and tumor penetration [2, 21]. The packaging of single or double stranded oligonucleotides or plasmids into nanoparticles (of less than 200 nm in size) facilitates the intratumor accumulation of the therapeutic agents due to enhanced permeability and retention effect (EPR), a phenomenon of passive crossing through the leaky tumor vasculature [21]. Moreover, several strategies for nanoparticles bioengineering have allowed the conjugation of targeting moieties onto the surface of nanoparticles

in order to increase tumor selectivity and the specific uptake in target tumor cells. This active targeting approach allows exclusive recognition, entrance and release of the therapeutic agent only in target cells, thus achieving effective blockade of cancer progression, while minimizing side effects in healthy cells and tissues [16]. Conventional nanomaterials used to deliver gene-silencing agents include lipid, polymer and inorganic nanoparticles, but different innovative nano-architectures are now being explored to increase efficacy.

2.2.1 Lipid-based nanovectors

Classical transfection systems for mammalian cells are based on cationic lipid formulations, which confer stability and favor the uptake of negatively-charged nucleic acids. Accordingly, most of the candidate non-viral anti-cancer gene therapeutic strategies involve formulation into lipid-based nanovectors, which include liposomes, stable nucleic acid lipid particles (SNALPs) and lipidoid nanoparticles [2, 22]. Gene silencing products with lipid nanoparticles have been widely exploited for siRNA delivery. Indeed, some lipid-based nanoparticles are currently in clinical trial, and novel cationic lipid molecules have been proposed for their ability to increase effectiveness [2, 20, 22-25]. Among them, SNALPs, made of ionizable cationic lipids as a core component, display huge siRNA encapsulation capability, together with the maintenance of uniform and small size. SNALPs composed of different lipid formulations have been developed for breast cancer therapy. These systems can package siRNAs directed toward key tumorigenic genes, such as polo like kinase 1 (PLK1), human protein-kinase N3 (PKN3), and β3 integrin [20, 25, 26]. In preclinical studies, effective suppression of tumor growth and onset of metastasis in mice bearing orthotopic xenografts of HER2-positive or triple negative breast cancer (TNBC) have been demonstrated [25, 27].

2.2.2 Polymer-based nanovectors

Non-lipid nanovectors contain chitosan, polyethylenimines (PEI), dendrimers, poly(lactic-co-glycolic acid) and other polymeric materials used as building blocks. Similarly, to most lipid-based

nanovectors, they generally function as polycation systems to induce the formation of nanosized complexes by electrostatic interactions with negatively charged nucleic acids [2]. Some cationic polymers have been extensively studied in recent years due to their versatility in structure and assembly, which allows efficient delivery, protection and intracellular release of nucleic acids [20, 26]. The strong positive charge of these vectors increases nucleic acid stability on one side, but can also promote colloidal instability of the final complex in physiological condition, inducing undesirable side effects such as increased cellular toxicity and non-specific accumulation *in vivo*. Therefore, modifications of these polymer-based complexes with hydrophilic polyethylene glycol (PEG), hydrogels, aliphatic lipids or small hydrophobic substitutions have been implemented to enhance colloidal stability of the complex, and improve biocompatibility and blood circulation time upon administration *in vivo* [28].

Achievement of complex stability is particularly relevant to develop vectors for RNA coupling. Indeed, siRNAs and miRNAs hold some unfavorable features, such as small molecular size and high rigidity, which hinder complex stability [29]. In this regard, the choice of the appropriate nanovector (*i.e.* molecular weight, structure, surface modification and charge) is essential for achieving therapeutic efficacy. To improve the *in vivo* delivery of therapeutic nucleic acids, combination of cationic polymer-based complexes with lipids has been deeply explored. This process involves the non-covalent modification of PEI polyplexes with phospholipids to obtain the innovative lipopolyplexes [28]. For their formation, the usage of neutral, anionic or PEG-coated phospholipids is suitable to reduce surface charges, and enhance transfection efficiency and colloidal stability. In particular, lipid-polymer nanoparticles have been evaluated as drugs delivery systems and for the treatment of multi-drug resistant breast cancer [30]

2.2.3 Inorganic nanoparticles

Different classes of inorganic nanoparticles have been explored as gene vectors due to their ability to promote oligonucleotides adsorption, concentration and protection [14]. Metallic nanoparticles,

carbon nanotubes, mesoporous silica and calcium phosphate nanoparticles are included in this category. Compared with lipid-based vectors, dendrimers or biodegradable polymers, inorganic nanoparticles present advantageous features, including high colloidal stability, tunable size and shape, and ease of surface functionalization [14]. However, their clinical application requires biocompatibility and the absence of antigenic activation, which is usually achieved by creating hybrid nanosystems with surfactants coating or lipid modifications. Genes can be loaded onto positively charged coatings, encapsulated into calcium phosphate nanoparticles by means of electrostatic interactions, or conjugated to cleavable linkers onto the nanoparticle surface [31-33].

Intrinsic properties of some inorganic materials can contribute to effectiveness of anti-cancer treatments. In case of magnetic nanoparticles, cancer cells transfection can be accelerated by applying an external magnetic field, which promotes phagocytosis of the genetic nano-agent through a process known as magnetofection [34]. Gold nanoparticles and nanorods are instead investigated for photothermal gene delivery, in which electrostatically bound DNA can be released from the complex under laser radiation without disruption of the genetic material [32, 35]. In recent years, calcium phosphate nanoparticles have been intensively reconsidered in DNA/RNA delivery for breast cancer therapy [31, 33]. As other cations, they can form stable ionic complexes with a considerable payload of nucleic acids, and possess high transfection capability and easy biodegradation. Major concerns that limited the use of these nanoparticles have been overcome by the coupling with polymeric systems, PEG or lipid shells, reducing aggregation and increasing nanoparticle stealth and cellular penetration [31-33]. Different strategies for inorganic nanoparticles functionalization with aptamers or other targeting ligands have greatly improved the selective targeting of breast cancer cells and transfection efficiency [17].

2.2.4 Oligonucleotide nanotechnology

In addition to the above-described nanovectors, nanosystems made of free nucleic acids as building blocks have also been proposed in some breast cancer settings with promising results [36]. They

consist of nanometer-scale architectures constructed via bottom-up self-assembly of DNA fragments and therapeutic RNA. In this class of nanosystems, the size of the complex, as well as the spatial orientation and density of cancer-targeting ligands on the nanoparticle surface can be finely controlled. Moreover, the sequence of RNA nanoparticles can be tuned to either induce an inflammatory response in the tumor microenvironment or achieve absence of immunogenicity [37, 38].

3. Gene therapeutic strategies

The wide range of delivery technologies now available have been explored in order to vehicle several classes of gene-based therapeutic agents. As distinct genetic alterations and gene expression profiles differentially affect the development and progression of breast cancer, correction of defective genes and regulation of gene expression through gene therapy has emerged as an innovative treatment strategy for breast cancer. Several approaches to induce genetic modification of a target cell have been evaluated, including transferring genes, segments of genes, or oligonucleotides such as siRNAs and miRNAs.

3.1 Gene replacement and gene addition

The more classical view in gene therapy for cancer and other diseases was represented by the replacement of mutated genes with their normal counterparts. Indeed, loss-of-function mutations in tumor suppressor genes have been identified as key events in breast cancer, with subsequent uncontrolled tumor progression and onset of metastasis. Therefore, the transfection of cancer cells with completely functional tumor suppressor genes (*e.g. TP53*) has been investigated as an anti-cancer strategy with both viral vectors and few nanosystems, resulting in inhibition of breast tumor growth and apoptosis induction [39, 40]. To obtain the death or growth arrest of cancer cells, another investigated strategy regards the transfection of suicide genes coding for toxins or drug-activating enzymes that convert prodrugs into cytotoxic compounds. In particular, the transfer of the

HSV-mediated thymidine kinase (TK) gene in combination with gancyclovir treatment, or the use of cytosine deaminase (CD) gene under the control of the HER2 promoter to induce breast tumor reduction upon conversion of the prodrug fluorocytosine into 5-fluorouracil (5-FU), have been successfully investigated in breast cancer clinical trials [18].

A quite distinct conceptual approach aims at reducing the severe toxic effects of chemotherapy, by promoting bone marrow protection. The transfer of drug resistant genes in hematopoietic stem cells ex vivo and subsequent autologous transplantation of transfected cells in patients will make chemotherapy more effective and tolerated even at higher dosage [41].

3.2 Alteration of gene expression

Beyond gene addition, silencing of gene expression is another therapeutic strategy that has revealed impressive impact on cancer therapy. The possibility to shut down oncogenes and key drivers involved in cancer initiation, progression, metastasis and angiogenesis by transfecting antisense oligonucleotides with specific sequence, has greatly contributed to increase the potential of cancer-targeted therapy (Table 1).

Since the discovery of the RNAi as a key post-transcriptional mechanism of gene expression regulation, siRNAs and short hairpin RNAs (shRNAs) have gained interest [22]. Their mechanism of function is perfect matching to target gene transcript and subsequent prevention of protein translation with high specificity. The ability of RNAi systems to virtually target any oncogenic process greatly enlarges the potential of targeted therapies, which was strictly limited to classical “druggable” targets. A number of siRNA targets are attractive for breast cancer treatment, including cell cycle regulators, signaling proteins, proto-oncogenes, players of epithelial-to-mesenchymal transition (EMT), angiogenic factors, and proteins associated with drug resistance. An overview of the most recent RNAi-based strategies evaluated in preclinical models of breast cancer is shown in Table 1. Over time, the identification of effective antisense and RNAi drugs has faced several challenges including toxicity, sensitivity to degradation, susceptibility to dissociation after systemic

injection, low cellular uptake, and lack of specificity. These challenges have been largely overcome by the introduction of modifications in the backbone of the oligonucleotides, making them chemically stable and less susceptible to degradation. Even though delivery systems still represent a limitation, the effectiveness of siRNA therapies has been extensively demonstrated, and some nanovectors have shown outstanding advantages for the delivery of these molecules [2, 8].

3.3 Epigenetic regulation

Although some genes have been discovered as key drivers of cancer initiation and progression, and targeted therapies have revealed impressive efficacy in tumor management, it is worth noting that targeting a single gene may be an inefficient long-term treatment strategy due to genetic instability and intrinsic heterogeneity of cancers. For this reason, another class of RNA molecules has drawn particular interest in the field of gene silencing and gene expression regulation (Table 1).

MiRNAs are single-stranded RNAs that promote gene expression regulation at the post-transcriptional level, by binding the target transcripts and repressing translation [73]. In both physiological and disease settings, these molecules play crucial roles in regulating cell cycle, proliferation, differentiation, metabolism and apoptosis [73]. Accordingly, the expression level of many miRNAs has been found altered in tissue and/or plasma of breast cancer patients [74-77]. Deregulation of miRNA levels has important implications in cancer, because a single miRNA can simultaneously affect a broad set of targets. Therefore, both miRNA mimics and miRNA inhibitors have been developed and successfully tested as therapeutic agents to achieve breast tumor regression. In particular, miRNA-based therapeutic strategies are now extensively explored in breast cancer preclinical models in order to regulate the expression of oncogenic genes and pathways and the interaction with the immune microenvironment, to modify the expression of surface markers, and to restore cancer cells sensitivity to therapy [33, 36, 42, 44, 62, 71, 74, 78]. This is particularly relevant for aggressive cancers that still lack effective drugs, such as TNBC, or in case of onset of resistance. The ability to selectively manipulate tumors and restore the cellular

equilibrium of deregulated miRNA expression profiles is now emerging as a promising clinical opportunity thanks to the impressive improvements in the development of non-viral nanocarriers, including the combination of miRNA-based therapy with other anticancer therapies, and the exploration of potential synergistic effects on tumor growth [8].

4. Prospective advancements for gene therapy in breast cancer

Anti-cancer treatments have several major limitations, including severe side effects and the emergence of drug resistance. The use of combination therapy involving gene therapy and agents that are currently the standard of care for breast cancer has been suggested as an innovative therapeutic strategy. However, conventional DNA- and RNA-based gene therapies pose great safety and efficiency challenges due to the risk of mutagenesis, delivery barriers, and transient and off-targets effects. Thus, further efforts are needed to identify and develop more effective systems to modify cancer-related genes and deregulated downstream pathways. Human mesenchymal stem cells (MSCs) have been proposed as potential innovative vehicles for gene therapy due to their self-renewing and tumor-homing ability, and low immunogenicity. Furthermore, advancement in genome engineering technologies, including the development of CRISPR/Cas9 systems has the potential to revolutionize clinical research and personalized treatments. Besides genetic mutations and gene expression aberrations, the host immune anti-cancer response has been deeply investigated for immunotherapy applications. Genetic engineering of autologous T lymphocytes or dendritic cells (DCs), and cancer vaccines represent powerful strategies to generate a specific anti-tumor immune response against a patient's cancer.

4.1 Combinatorial treatments

Treatment strategies in breast cancer are based on clinicopathological and molecular classification [79]. While hormonal therapy and anti-HER2 monoclonal antibodies have substantially improved the prognosis of luminal and HER2-positive cancers, chemotherapy remains the mainstay of

treatment for TNBC. Furthermore, chemotherapy remains important in combination with targeted therapies in the metastatic setting. Current treatments, in particular chemotherapy, have several limitations, including long-term toxic effects, lack of specificity for cancer cells, and emergence of therapy resistance. Furthermore, the inherent genomic and molecular heterogeneity of breast tumors make cancer treatment particularly challenging. Thus, the development of novel strategies to increase the effectiveness of current therapy, reduce side effects, and reverse treatment resistance is an urgent clinical need.

The efficiency of combinatorial treatments is demonstrated by the approval of antibody-conjugated chemotherapeutics, such as trastuzumab emtansine (T-DM1), for the treatment of late-stage HER2-positive breast cancer. In this context, the combination between standard anti-cancer treatments and gene therapy is emerging as a promising approach for the treatment of cancer. Besides their direct therapeutic application, monoclonal antibodies can be used to confer target selectivity to a wide range of nanoparticles [17]. For instance, trastuzumab functionalization of nanocomplexes enveloping DNA or RNA molecules, including siRNA against B-cell lymphoma-extra large (*BCL-XL*), CXC chemokine receptor 4 (*CXCR4*), *HER2*, *PLK1*, and signal transducer and activator of transcription-3 (*STAT3*), can be used for the specific targeting of *HER2*-overexpressing breast cancer cells, resulting in an effective therapeutic strategy that combine targeted and gene therapies [27, 37, 47, 80, 81].

Even though several nanoparticles-based chemotherapeutic drugs, including liposomal doxorubicin, taxanes and cisplatin, are approved or are undergoing clinical trials for breast cancer, monoclonal antibodies can be used as a molecular tag to specifically deliver chemotherapeutics drugs into cancer cells [16, 17, 32, 82]. A potential limitation of this latter approach, especially for TNBC, is represented by the lack of cell-specific markers beyond estrogen receptor (ER) and HER2 in breast cancer. Importantly, combination treatments of conventional chemotherapy and antisense oligonucleotides targeting genes responsible of multi-drug resistance (*e.g. MDR1*) or cancer stem cells (*e.g. ALDH1, CD44*) have been evaluated in breast cancer [18, 19, 83].

4.2 Therapeutic stem cells

Human MSCs are self-renewing multipotent cells. These stem cells properties, together with the tumor-homing ability, immunomodulatory functions, and low immunogenicity made stem cell-based systems an attractive approach for cancer therapy. However, one of the most interesting approaches is the use of MSCs as novel delivery vehicles for anti-cancer drugs or genetic materials to primary or metastatic cancers. In particular, MSCs have been used for suicide gene therapy, involving the introduction into tumor cells of a transgene that induce cancer cell death [84]. For instance, the use of MSCs as vehicles for the delivery of tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) or oncolytic viruses expressing the sodium iodine symporter demonstrated significant anti-tumor effects in *in vivo* models of breast cancer [84].

It is worth noting that MSCs have a well-known role in tumor initiation, progression, and resistance to therapies [85]. Furthermore, MSCs have been also demonstrated to be involved in local feedback loops with the tumor immune microenvironment, supporting aggressiveness and metastatic dissemination in breast cancer [85]. These tumor-promoting functions have raised concerns about the potential clinical application of MSCs for gene therapy. Indeed, several strategies have been proposed to target the tumor MSC pools, including suicide gene therapy, and immunostimulatory approaches [85].

4.3 Genome editing

Over the past two decades, different genome engineering technologies have been extensively used for research purposes to understand the role of specific genes in cancer development, progression and therapeutic response [3]. Recent advancement in the genome editing systems has led to a significant increase of the efficiency and specificity of gene targeting using site-specific endonucleases, including ZFNs, TALENs, and CRIPR/Cas9 [2]. ZFPs and TALENs, which are composed of a customized DNA-binding module and a non-specific DNA cleavage domain, can

generate multiple genetic modifications by inducing DNA double-strand breaks (DSBs) that stimulate the error-prone non-homologous end joining or the more specific homologous recombination pathways [3, 74]. Even though these genome engineering methods may partly overcome the limitations of RNAi techniques, their therapeutic application is restricted by the complexity of designing the custom endonucleases. More recently, there has been growing interest on the more versatile RNA-guided CRISPR/Cas9 system, which has the potential to revolutionize the field of genome engineering for personalized medicine by overcoming many of the limitations of other editing methods. Basically, the DNA endonuclease Cas9 can be specifically guided to a target site using a single-guide RNA (sgRNA), inducing DSBs that initiate the DNA repair processes [3]. Stable Cas9-mediated gene modification can be accomplished through the transient transfection of plasmid DNA encoding Cas9 endonuclease and sgRNA, or through the stable deliver of CRISPR components using viral vectors [3]. The efficiency of this system allows the rapid and powerful engineering of human stem and cancer cells, as well as components of the immune system *in vivo* and *ex vivo* for therapeutic purposes.

Gene expression profiles and novel sequencing-based technologies have increased our knowledge of genetic aberrations and deregulated oncogenic pathways, which, together with the host immune response, affect breast cancer development and progression [86]. Several recurrent mutations and a wide range of rare mutations in oncogenes and tumor suppressor genes, including *BRCA1/2*, *GATA3*, *MAP3K1*, *MLL3*, *PIK3CA*, *PTEN*, *RB1*, and *TP53*, have been found with different frequencies in breast cancer subtypes [86]. These loss-of-function or gain-of-function mutations can be targeted by the CRISPR/Cas9 system to restore the physiological function of each gene and downstream pathways. Furthermore, CRISPR/Cas9 has also the potential for epigenome and gene expression modulation through the specific targeting of deregulated miRNAs [87, 88].

In recent years, considerable research efforts have focused on the relevance of host immune anti-cancer response and on the opportunity for immunotherapy applications in breast cancer [89]. In particular, increasing attention has been paid to the blockade of inhibitory pathways, such as the

programmed death 1 (PD-1)/PD-L1 signaling [89]. Beyond the development of specific anti-PD-1/PD-L1 antibodies, the CRISPR/Cas9 system has been used to genetically modify the PD-1 locus in T cells, leading to increased T cell effector activity [88]. Furthermore, the genetic engineering of autologous tumor-specific T cells for adoptive T cell therapy can be efficiently achieved with the CRISPR/Cas9 system [88]. In this regard, genetic engineering allows the modification of T cell's specificity toward a patient's cancer by introducing a cloned T cell receptor (TCR), or a chimeric antigen receptor (CAR), as discussed in the next paragraph [90].

4.4 Immunomodulatory therapies

Beyond the growing interest in directly targeting the immune system through the blockade of inhibitory pathways, such as the PD-1/PD-L1 or the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) pathways in breast cancer, the genetic engineering of autologous T lymphocytes or dendritic cells (DCs), and cancer vaccines represent powerful strategies to generate a specific anti-tumor immune response against a patient's cancer (Figure 2).

Using ex vivo adoptive gene transfer, T cells can be genetically modified to express a novel TCR or a CAR to specifically recognize a tumor-associated antigen. The TCR interacts with an antigenic peptide presented by the human leucocyte antigen (HLA) class I complex, and induces the T cell-mediated cytolysis of the target cell (Figure 2) [91]. Virtually, TCRs have the potential to target all cancer-associated antigens, including mutated polypeptides from endogenous proteins. Although considerable efforts have been directed towards the development of efficient TCR engineering strategies, the frequent down-regulation of HLA class I during tumor progression, the requirement compatibility between HLA haplotype of the patient and the TCR, and disappointing results from early clinical trials have reshaped research directions [1, 91]. A CAR is a transmembrane molecule, which combines an extracellular tumor-specific fragment from a monoclonal antibody with intracellular signaling motifs capable of boosting T cell activation [91]. In contrast to the TCR, upon the binding to a tumor-associated antigen through its antibody moiety,

the CAR induces apoptosis in the target cell independently of HLA, allowing the recognition of targets other than peptides, and a broader selection of patients eligible for adoptive therapy (Figure 2) [90]. However, CAR T cell-based therapy requires cancer-related targets to be cell surface antigens, thus excluding the recognition of all mutated intracellular proteins [91].

Another approach to potentiate anti-cancer immune response is the use of cancer vaccines, which provide the patient's immune system with specific tumor antigens. Therapeutic cancer vaccines can derive from whole tumor cells, protein antigens, peptides, DNA, or DCs (Figure 2) [92-94]. Whole-cell vaccines are able to induce an immune response against multiple tumor targets [94]. Clinical trials evaluating cell-based vaccines failed to show a consistent clinical benefit in cancer patients, although demonstrating the development of an immune response [94, 95]. However, different strategies other than the administration of intact cancer cells are currently being explored, including genetic engineering of tumor cells and fusion with DCs [93, 94]. For instance, autologous whole tumor cells have been modified to express granulocyte-macrophage colony-stimulating factor (GM-CSF) and to downregulate transforming growth factor (TGF)-β to enhance the recruitment of DCs and boost the activation of anti-tumor immune response [95].

As any effective anti-tumor immune response involves the processing and presentation of cancer-related antigens by DCs, these cells have been proposed to be crucial for the development of an efficient vaccination strategy. Beyond the use of viral vectors, DCs have been explored as a delivery vehicle with inherent adjuvant properties (Figure 2). Indeed, DCs derived from circulating blood precursors can be loaded with tumor antigens or peptides, transfected with tumor-derived RNA or DNA, or genetically engineered to produce a specific tumor antigen, and then infused back to the patient, thus serving as a delivery vehicle with inherent adjuvant properties [93, 94, 96]. This approach has demonstrated clinical efficacy in certain types of cancer, leading to the approval of a DC-based vaccine for the treatment of metastatic hormone-refractory prostate cancer [96]. However, there are challenges related to the use of DC-based vaccine, including the complexity of *ex vivo* manipulation, and the existence of multiple DCs subsets with different ability to process and

present antigens and to modulate the activation of T cells [94, 96]. Although several strategies are being evaluated to improve the effectiveness of DC-based vaccines, including the appropriate selection of DCs subset and adjuvants, and the enhancement of DCs functionalities, their clinical utility is still unclear [94, 96].

Finally, active immunotherapies can also take advantage from the use of protein antigens, peptides and nucleic acids from tumor cells (Figure 2) [92-94]. Several strategies for the delivery of genes encoding tumor antigens able to elicit a specific immune response have been described [92]. Advancement for the implementation of delivery systems, development of adjuvants and other immune stimulatory signals, have led to the evaluation of DNA-based vaccines in clinical trials for different diseases, including breast cancer [92]. In addition, peptides derived from tumor-associated antigens, most of which are based on wild-type protein sequences overexpressed by cancer cells, can directly bind to HLA molecules, thus overcoming the need for antigen processing and presentation, and allowing an efficient activation of T cells [94]. Despite these promising assumptions, single peptides demonstrated limited efficacy in clinical studies, and the addition of enhancers of immune response (*e.g.* IL-2) are being investigated [94].

A basic concept to highlight is that the activity and therapeutic efficiency of all immune-based gene therapies are dependent on the recognition of a specific tumor target. Ideally, the selected antigen would be non-essential to normal human tissues and expressed only by cancer cells. However, the identification of selective cancer antigens is challenging. Accordingly, therapies with CAR and engineered TCR are often associated with significant toxicities due to the recognition of the antigen expressed on normal tissues [90]. In this regard, neoantigens may represent valuable targets, as they are exclusive to tumor cells and arise from recurrent driver mutations that are crucial for the malignant phenotype [90].

5. Gene therapies under clinical evaluation for breast cancer

The majority of clinical trials in gene therapy (64.4%) have been undertaken in cancer patients [97]. A broad spectrum of delivery systems, including viral vectors, inorganic nanoparticles, and lipid- or polymeric-based nanovectors has been investigated. Furthermore, a wide range of strategies, including replacement gene therapy, oncolytic virotherapy, suicide gene therapy, RNAi, and immunotherapy has been evaluated. Even though several major challenges must still be addressed, clinical trials of gene therapy are beginning to show an improved safety profile and substantial therapeutic benefits. A growing number of clinical trials of gene therapy and immune-based therapy are being conducted for the treatment of breast cancer (Table 2).

The concerns about the safety of virus-based approaches led to a substantial decline of their application in clinical trials [97]. However, two clinical trials of *TP53*-targeting therapy in combination with chemotherapy, and a phase 1/2 study of Rexin-G, consisting of a viral envelope carrying cyclin G1 gene, have been recently completed in advanced or metastatic breast cancer, and their results are awaited (Table 2). Moreover, talimogene laherparepvec and JX-594 oncolytic viruses, and the retroviral vector Toca 511 for suicide gene therapy, are now being evaluated for triple negative and advanced/metastatic breast cancer (Table 2).

A growing interest is now focused on immunomodulatory therapies for breast cancer (Table 2). This is reflected by the evaluation of two anti-HER2 vaccines – NeuVax and AE37 – in phase 2-3 studies for the treatment of HER2-positive breast cancer (Table 2). Viral vectors are also emerging as important delivery systems for immunotherapy in breast cancer. Recent results from a randomized, phase 2 clinical trial suggested that the combination of a recombinant vaccinia virus producing the carcinoembryonic antigen (CEA) and mucin-1 (MUC-1), together with the triad of costimulatory molecules B7.1, ICAM-1 and LFA-3 (TRICOM), with docetaxel might provide a clinical benefit in metastatic breast cancer [98]. Viral vectors encoding a wide range of proteins, including TP53, CEA, IL-12, HER2, the EMT driver brachyury, and a fusion protein of human MUC-1 and CD40 ligand, are currently under clinical investigation for the boosting of anti-tumor immune response (Table 2). Furthermore, plasmid DNA has also been employed for cancer vaccine

and immunotherapy. For instance, plasmids DNA encoding CYP1B1, IL-12, mammaglobin-A, PRAME and PSMA, are in early clinical evaluation for breast cancer treatment (Table 2). Engineered TCRs and CARs are also being evaluated in clinical trials for their ability to "educate" the host immune systems to recognize specific tumor antigens, including CD133, CEA, EpCAM, HER2, mesothelin, MET proto-oncogene, NY-ESO-1, ROR1, and TP53 (Table 2). Furthermore, DCs-based vaccination strategies are under clinical evaluation for the treatment of breast cancer (Table 2). All these strategies are often combined with the administration of immunostimulatory cytokines, including IL-2, GM-CSF, and granulocyte colony-stimulating factor (G-CSF), to boost their anti-tumor activity (Figure 2).

Overall, siRNA- and miRNA-based therapies have not yet entered the clinic, with only one study evaluating RNAi technology in breast cancer (Table 2).

6. Expert opinion

Gene therapy has certainly the potential to impact clinical research. However, major limitations must still be overcome. In particular, the safety and efficiency of delivery systems should be improved by further engineering their composition, immunogenicity, and functionalization, and by developing novel strategies to potentiate their specificity toward cancer cells. Furthermore, the current efficiency of these systems may be sub-therapeutic for certain diseases, and off-target genomic alterations may cause detrimental effects. Thus, the rate and specificity of gene modification must be increased before considering gene therapy as a concrete option for the treatment of cancer patients. Beyond virus-based approaches, nanoparticles have several advantages, including the capability to carry a superior and more varied drug cargo, and the opportunity of being differently functionalized. These versatile systems have thus a great potential to be further engineered and to be used for combination treatment between conventional anti-cancer drugs and gene-based therapy. MCSs deserve additional consideration as novel delivery vectors, due to their tumor-homing ability and low immunogenicity. However, the tumor-promoting

functions of MSCs, as well as their involvement in therapy resistance, must be taken into account, especially in several types of cancer, such as a subgroup of TNBC. In particular, the relationship between MSCs functions and the tumor immune microenvironment is emerging as a crucial factor driving tumor development, progression and therapy resistance. Thus, additional studies are required to understand the role of MSCs in cancer tissues, and to identify any oncogenic change that can potentially occur following their engraftment at the tumor site.

Even though gene therapy was classically envisioned as an option to treat a disease at a genetic level, further efforts are also required to boost the development of RNAi technologies, and to increase the number of siRNA- and miRNA-based therapeutics being tested in clinical studies, ultimately translating this knowledge into clinical practice. It is also worth noting that each cancer is a unique genomic entity, which can carry different types of mutations on the same gene, and a combination of multiple distinct mutated genes and noncoding regions. Such a complex landscape can differentially impair multiple cancer-related pathways. In this context, advancement in genome editing technologies, such as CRISPR/Cas9 system, can be used to specifically modify multiple different sites simultaneously, with the potential to reshape research directions and improve the clinical management of cancer patients. However, several technical challenges must still be addressed, and the functional relevance of each mutations, as well as their overall effects on signaling pathways should be deeply dissect before considering genome editing techniques as potential therapeutic strategy in humans.

Besides conventional gene therapy approaches, immune-based approaches have received increasing attention as new therapeutic options for patients with breast cancer, especially HER2-positive and TNBC. Immunomodulation of cancer cells and the tumor microenvironment trough the selective delivery of cytokines and chemokines, as well as the development of cancer vaccine to boost anti-tumor immunity have been evaluated in preclinical and clinical studies of breast cancer, proving evidence of immune activation. However, substantial evidence of clinical activity is still lacking. Therapeutic cancer vaccines and modified TCRs and CARs have proven to be challenging

irrespective to the engineering strategy employed, the type of antigen used, and the supportive cytokine regimen provided. Noteworthy, several types of cancer, including breast cancer, have not been traditionally considered an immunogenic tumor. In breast cancer, there is a paucity of both antigens specific of cancer cells, and neoantigens due to the reduced number of recurrent mutations in cancer-related genes. Expanding the use of immune-based therapies to treat cancer patients will require major technical improvements, the identification of antigen specific of cancer cells, and further knowledge on the biological mechanisms underlying immune editing, antigen processing and presentation, and long-term effects of anti-cancer immune response.

Article highlights box

- Gene therapy has a great potential to improve the clinical management of breast cancer patients.
- Further efforts are required to increase the clinical application of RNAi-based therapeutics.
- Genome editing and stem cell-based systems, may certainly contribute to translate gene therapy into clinical practice.
- Immune-based strategies is emerging as an attractive therapeutic opportunity for specific breast cancer patients.
- Technical challenges need to be addressed before using gene therapy as a concrete option for the treatment of breast cancer.

List of abbreviations: CRISPR/Cas9: clustered regularly interspaced short palindromic repeat /associated protein 9; DCs: Dendritic cells; EMT: epithelial-to-mesenchymal transition; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HER2: erb-b2 receptor tyrosine kinase 2; HSV-1: herpes simplex virus type 1; IL-2: Interleukin-2; MAPK: mitogen-activated protein kinase; MDR1: Multidrug resistance gene 1; miRNA: microRNA; MSCs: mesenchymal stem cells; PEG: polyethylene glycol; PEI: polyethylenimines; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; RNAi: RNA interference; siRNA: small-interfering RNA; TALEN: transcription activator-like effector nuclease; TCR: T cell receptor; TNBC: Triple negative breast cancer; ZFN: zinc-finger nuclease;

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Declaration of interest

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Table 1. Recent preclinical advances in nanotechnology for RNA-based therapy in breast cancer

Nanoparticle type	Therapeutic agent	Target gene	Therapeutic effect	Nanocompound	Reference
Lipid nanoparticles	eIF4E-siRNA	Eukaryotic translation initiation factor 4E	Resensitization of triple negative breast cancer to paclitaxel	Dual pH-sensitive RGD-PEG(HZ)-ECO/sieIF4E	[24]
	miR-125a	HER2 proto-oncogene and other miR-125a targets	Suppression of PI3K/AKT and MAPK hyperactivated signaling pathways, cellular proliferation and migration potential of HER2-positive metastatic breast cancer cells	HA-coated lipid nanoparticle platform	[42]
	β3 integrin-siRNA	β3 integrin	Attenuation of EMT and invasion; inhibition of 3-D organoid growth; metastasis inhibition in triple negative breast cancer *in vivo*	RGD-targeted lipid-ECO-based nanoparticles (ECO/si β3)	[25]
Liposomes	EGFR-siRNA	EGFR	Inhibition of MCF-7 breast tumor growth *in vitro* and *in vivo*	siEGFR/protamine/CS-encapsulated T7-PEG-DSPE-coated cationic liposomes (T7-LCP/siEGFR nanoparticle)	[43]
	Anti-miR-21, anti-miR-10b	miR-21 and miR-10b targets	Reduction of triple negative breast cancer cells migration *in vitro*; reduction of tumor growth *in vivo*	uPA receptor-targeted antisense-loaded PLGA-b-PEG polymer nanoparticles	[44]
	Antisense (AS) against OPN and BSP	OPN and BSP	Decrease in tumor bone metastasis incidence and size in breast cancer rat model	AS-PLGA nanoparticles	[45]
	Anti-miR-21, 4-hydroxytamoxifen	MiR-21 targets	Antiproliferative and apoptotic effects in ER+ breast cancer *in vitro*	4-OHT and anti-miR-21 co-loaded PLGA-b-PEG nanoparticles	[46]
	siRNA	BCL-XL and STAT3	Suppression of oncogenes in HER2+ cancer cells	pH-sensitive siRNA-loaded endosome-disruptive biotinylated co-polymeric nanocarrier	[47]
Polymeric nanoparticles	MDR1-siRNA, doxorubicin	MDR1	Reversion of multi-drug resistance and inhibition of tumor growth *in vivo*; reduction of doxorubicin toxicity in non-target tissues	FA-decorated PEG-b-(PCL-g-PEI)-b-PCL triblock co-polymeric nanomicelles	[48]
	BMI-1-siRNA	BMI-1	Reduction of BMI-1 expression in MCF-7 breast cancer cells *in vitro*; tumor suppression *in vivo*	PEI-laminarin conjugate nLP/siBMI-2	[49]
	ABCG2-siRNA	ABCG2	Gene silencing efficiency and enhancement of adriamycin susceptibility in MCF-7/ADR-resistant xenograft tumors *in vivo*	mPEG-PLGA-PLL (PEAL) nanoparticles with ultrasound-targeted microbubble destruction	[50]
	TWIST1-siRNA	TWIST1 and EMT-related target genes	Reduction in migration and invasion *in vitro*; long-lasting siRNA delivery in xenograft orthotopic tumors	PAMAM dendrimer complexes	[51]
	MDR-shRNA, doxorubicin	MDR1	Increase of the tumor suppression effect of doxorubicin in MCF-7/adriamycin cells; reduction of doxorubicin efflux	Doxorubicin-PLGA/PEI/ MDR1-shRNA nanobubbles	[52]
	MiR-145 plasmid	MiR-145 and its targets	Cytotoxicity effect in MCF-7 cells *in vitro*	MiR-145 chitosan polyplex nanoparticles	[53]

	AURKA-siRNA, paclitaxel	AURKA	Synergistic antitumor efficacy of the two drugs in MDA-MB-231 breast cancer *in vitro* and *in vivo*	HA-based OA-g-bPEI-conjugated redox-sensitive micellar system (HSOP)	[54]
	RHOC-siRNA	RHOC GTPase	Inhibition of invasion, motility and migration of MDA-MB-231 cells *in vitro*	Degradable, pH-sensitive β-cyclodextrin-based polymeric carrier (smart-anti-RHOC particles)	[55]
	SNAIL and TWIST-siRNA, paclitaxel	SNAIL, TWIST	Inhibition of tumor growth and metastasis in metastatic breast cancer *in vivo*	PEI-PDHA/PEG-PDHA/PTX/siSna/siTw)	[56]
	VEGF-siRNA, doxorubicin	VEGF	Antiproliferation and inhibition of tumor spheroids *in vitro*; decrease in tumor microvessel density and doxorubicin toxicity *in vivo*; increase in life span in mice	FA-decorate pH-responsive PHD/PPF/siVEGF nanocomplex	[19]
	MDR1-siRNA	MDR1	Overcoming multidrug resistance in adriamycin-resistant breast cancer cells *in vitro* and *in vivo*	MDR1-targeted poly-siRNA/Thiolated glycerol chitosan nanoparticles (psi- MDR1-tGC NPs)	[57]
	BCL2-siRNA, doxorubicin	BCL2	Efficient delivery and cytotoxicity effect in MCF-7 cells *in vitro*	FA-decorated hydrophilic cationic star-block terpolymer nanomicelleplexes (PGAH-b-PDMAPMA)3-g-PEG	[58]
	MYC shRNA	MYC	Tumor growth arrest and increased survival in mice bearing BRCA2/TP53-mut mammary tumors	PEI-grafted PGMA platform	[59]
	IL17RB-siRNA, doxorubicin	IL17RB	Significant effect on doxorubicin -induced cytotoxicity, apoptosis induction and migration inhibition in MDA-MB-361 cells *in vitro*	Carboxymethyl dextran chitosan nanoparticles (doxorubicin -siRNA-CMD-Ch NPs)	[60]
	CD73-siRNA	Ectonucleotidase molecule CD73	Suppression of CD73 in 4T1 breast cancer cells *in vitro* for immunotherapy	CD73-siRNA-loaded chitosan-lactate nanoparticles	[61]
	Anti-miR-10b LNA	BMI and other miR-10b targets	Induction of apoptosis, inhibition of proliferation and reduction in invasion and migration *in vitro*; regression of lymph node metastasis *in vivo* in combination with low-dose doxorubicin	Dextran coated magnetic nanoparticles (MN-anti-miR-10b)	[62]
Inorganic nanoparticles	AKT1-siRNA	AKT1	Inhibition of cell cycle progression and induction of apoptosis in MCF-7 cells *in vitro* and *in vivo*	Amorphous calcium carbonate hybrid nanospheres functionalized with CaIP6 composite nanoparticles (ACC/CaIP6/siAKT1)	[31]
	Survivin-siRNA, tamoxifen	Survivin	Enhancement of breast cells death *in vitro*	Arginine-functionalized gold nanoparticles-stabilized capsules (NPSC/siSurv/drug)	[63]
	AKT-siRNA, thymoquinone	AKT	Apoptosis induction in MCF-7 cells by induction of p53 *in vitro* and *in vivo*; overcoming therapy resistance	Multilamellar gold niosome containing thymoquinone and AKT-siRNA (si-RNA-Nio-Au- thymoquinone)	[64]
Hybrid nanoparticles	MiR-221/222 inhibitors (miRi), paclitaxel	MiR-221/222 targets	Enhancement of paclitaxel efficacy on triple negative breast cancer *in vitro*	Lipid-coated calcium phosphate-polymer hybrid nanoparticles/miRi/pac	[33]
	CXCR4-siRNA	CXCR4	Reduction of CXCR4 expression in MDA-MB-231 breast cancer *in vitro* and *in vivo*	siRNA-loaded pH-responsive hybrid nanogel particles	[65]
	BMI1 and hTERT-siRNA	BMI1 and hTERT	Gene silencing efficiency and tumor growth inhibition *in*	Lipo/PEI/siRNA nano-condensate	[66]

			vitro		
	HER2-siRNA	HER2, delta16 HER2	Reduction of tumor proliferation in trastuzumab-resistant breast cancer *in vitro* and *in vivo*; non-toxic effect in non-tumorigenic cell lines *in vitro*	PEI-PEG-modified trastuzumab -conjugated mesoporous silica nanoconstructs (T-siHER2-NP)	[67]
	VEGF-shRNA	VEGF	Inhibition of VEGF gene expression in MCF-7 cells *in vitro*; potential in cancer detection by magnetic resonance imaging	Multifunctional PEI-grafted Fe3O4@SiO2/BEGF shRNA nanocomposites	[68]
	VEGF-siRNA, captopril	VEGF	Antiangiogenic and antitumor activity in MDA-MB-435 tumor xenografts	Captopril-PEI-conjugated Gold nanoparticles (siRNA/CP/GNP)	[69]
	Survivin-siRNA	Survivin	Apoptosis induction in MCF-7 breast cancer cells *in vitro*	Polyacrylate-PEI-covered Fe3O4 nano-sized particles (Fe3O4-PA-PEI NP)	[70]
	Anti-miR-21	MiR-21 targets	Repression of triple negative breast cancer growth at low doses	Triple functional phi29 pRNA-3WJ nanoparticles	[36]
RNA nanostructures	MiR-205 mimic, miR-221 antagomiR	MiR-205 and miR-221 targets	Long-lasting inhibition of triple negative breast cancer progression in vivo and survival advantage	Dendrimers-conjugated self-assembled RNA-triple helix hydrogel scaffolds	[71]
	Survivin and stathmin-siRNA	Survivin and stathmin	Inhibition of proliferation in MDA-MB-453 cells in vitro	LNA-modified chimeric aptamers	[72]

ABCG2: ATP binding cassette subfamily G member; AURKA: Aurora Kinase A; BMI-1: proto-oncogene, polycomb ring finger; BSP: bone sialoprotein; CXCR4: C-X-C motif chemokine receptor 4; EpCAM: epithelial cell adhesion molecule; EMT: epithelial-to-mesenchymal transition; FA: Folic acid; HER2: erb-b2 receptor tyrosine kinase 2; hTERT: human telomerase reverse transcriptase; IL17RB: interleukin 17 receptor B; MAPK: mitogen-activated protein kinase; MDR1: multidrug resistant protein 1; MYC: v-myc avian myelocytomatosis viral oncogene homolog; OPN: osteopontin; RHOC: ras homolog family member C; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; SNAIL: snail family transcriptional repressor; TP53: tumor protein p53; TWIST: twist family bHLH transcription factor; VEGF: Vascular endothelial growth factor;

Table 2. Gene therapies in clinical trials for the treatment of breast cancer

Vehicle and strategy	Study design	Breast cancer population	Other drugs	Estimated enrollment	Status	Study ID[a]
Viral-based strategies						
Oncolytic measles virus encoding thyroidal sodium iodide symporter	Open label, phase 1 safety study	Metastatic breast cancer	-	30	Recruiting	NCT01846091
Oncolytic therapy with a modified vaccinia poxvirus engineered by addition of the GM-CSF gene and deletion of the TK gene (JX-594)	Randomized, open label, phase 1/2 pharmacokinetics/dynamics study	Advanced breast cancer	Cyclophosphamide	118	Recruiting	NCT02630368
Talimogene laherparepvec (HSV-1) for oncolytic therapy	Open label, phase 1/2 efficacy study	Triple negative breast cancer, inflammatory breast cancer or breast cancer with inoperable recurrence	Neoadjuvant paclitaxel	46, 35	Not yet recruiting, Recruiting	NCT02779855, NCT02658812
Virus-based introduction of *MDR1* into autologous peripheral blood progenitor cells	Phase 2 safety/efficacy study	Metastatic breast cancer	Methotrexate, leucovorin, 5-FU, cyclophosphamide, thiotepa, paclitaxel, doxorubicin	42	Completed	NCT00001493
Suicide gene therapy with the retroviral vector Toca 511 encoding CD and the Toca FC prodrug 5-FC	Open label, phase 1 safety/efficacy study	Locally advanced or metastatic breast cancer	-	26	Recruiting	NCT02576665
Adenovirus-mediated delivery of *TP53*	Open label, phase 1/2 study	Locally advanced breast cancer	Doxorubicin, docetaxel	20, -	Completed	NCT00004038, NCT00044993
Retrovector bearing a dominant negative cyclin G1 construct (Rexin-G)	Open label, phase 1/2 safety/efficacy study	Recurrent and metastatic breast cancer	-	20	Completed	NCT00505271
Non-viral based strategies						
Transferrin targeted cyclodextrin-containing polymer carrying siRNA against RRM2	Open label, phase 1 safety study	Refractory breast cancer	-	24	Completed	NCT00689065
Immunomodulatory strategies						
5T4-modified vaccinia ankara vaccine	Open label, phase 2 study	Advanced breast cancer		-	Not yet recruiting	NCT00227474

TP53-modified vaccinia ankara vaccine	Open label, phase 1 safety study	Triple negative breast cancer	Pembrolizumab	12	Recruiting	NCT02432963
Recombinant vaccinia-CEA-MUC-1-TRICOM vaccine	Open label, phase 1 safety study	Advanced breast cancer	Sargramostim (GM-CSF)	51	Ongoing	NCT00088413
Vaccination with the Venezuelan equine encephalitis virus expressing HER2	Open label, phase 1 safety study	HER2-positive breast cancer	-	22	Ongoing	NCT01526473
Brachyury-modified vaccinia ankara and TRICOM vaccine	Open label, phase 1 safety study	Metastatic breast cancer	-	38	Ongoing	NCT02179515
Vaccination with an adenovirus-encoding for *HER2*	Open label, phase 1 safety/efficacy study	Locally advanced and metastatic HER2-low-expressing breast cancer	-	30	Not yet recruiting	NCT02751528
Vaccination with an adenoviral vector encoding a fusion protein of human MUC-1 and CD40 ligand	Open label, phase 1 safety study	Breast cancer	-	24	Recruiting	NCT02140996
Immunotherapy with an adenoviral vector engineered for the expression of human recombinant IL-12 under the control of the RheoSwitch Therapeutic System (RTS)	Open label, phase 1/2 safety/efficacy study	Locally advanced or metastatic breast cancer	Veledimex,	40	Recruiting	NCT02423902
Vaccination with a plasmid DNA encoding mammaglobin-A	Open label, phase 1 safety study	Metastatic breast cancer	-	15	Completed	NCT00807781
Vaccination with a plasmid DNA encoding CYP1B1 encapsulated in biodegradable microparticles	Open label, phase 1 safety/efficacy study	Advanced breast cancer	Cyclophosphamide	22	Completed	NCT00381173
Plasmid-based vaccination strategy targeting multiple antigens of cancer stem cells	Open label, phase 1 safety study	Advanced and recurrent HER2-negative breast cancer	-	30	Recruiting	NCT02157051
Local injection of a DNA plasmid encoding IL-12	Open label, phase 1 pharmacodynamics study	Triple negative breast cancer	-	10	Recruiting	NCT02531425
Vaccination with a DNA plasmid encoding *IGFBP2*, *HER2*, and *IGF1R*	Open label, phase 1 safety study	Non-metastatic, node positive, HER2-negative breast cancer	Sargramostim (GM-CSF)	30	Recruiting	NCT02780401
Vaccination with a plasmid DNA encoding *HER2*	Open label, phase 1 safety/efficacy study	Advanced and metastatic HER2-positive breast cancer	Sargramostim (GM-CSF)	66	Ongoing	NCT00436254
Personalized polyepitope DNA vaccine	Open label, phase 1 safety	Triple negative breast cancer	-	15	Recruiting	NCT02348320

	study					
Vaccination with a DNA plasmid encoding PRAME and PSMA	Randomized, open label, phase 1 safety/efficacy study	Breast cancer	-	12	Completed	NCT00423254
Immunogenic RNA vaccines	Open label, phase 1 safety/efficacy study	Triple negative breast cancer	-	30	Recruiting	NCT02316457
Vaccination with a HER2-targeted peptide (NeuVax)	Randomized, single blind, phase 2 efficacy study	Low and intermediate HER2-expressing breast cancer, high-risk HER2-positive breast cancer	Sargramostim (GM-CSF), trastuzumab	300, 100	Recruiting	NCT01570036, NCT02297698
Vaccination with a HER2-targeted peptide (NeuVax)	Randomized, double blind, phase 3 safety/efficacy study	Low and intermediate HER2-expressing breast cancer	Sargramostim (GM-CSF)	700	Ongoing	NCT01479244
Vaccination with a HER2-targeted peptide (AE37)	Randomized, single blind, phase 2 efficacy study	Node-positive and high-risk node-negative HER2-positive breast cancer	Sargramostim (GM-CSF)	600	Ongoing	NCT00524277
Vaccination with four tumor-associated peptides	Open label, phase 1 safety/efficacy study	Triple negative breast cancer	Durvalumab	20	Recruiting	NCT02826434
Vaccination with four tumor-associated peptides (PVX-410)	Open label, phase 1 safety/efficacy study	Triple negative breast cancer	Durvalumab	20	Recruiting	NCT02826434
Combination of two chimeric (trastuzumab-like and pertuzumab-like) HER-2 B cell peptide vaccine linked to a promiscuous T cell epitope from the measles virus fusion protein	Open label, phase 1 safety/efficacy study	Advanced HER2-positive breast cancer	-	36	Recruiting	NCT01376505
Vaccination with autologous breast cancer cells engineered to secrete GM-CSF	Open label, phase 1/2 safety/efficacy study	Metastatic breast cancer, Operable Breast Cancer	-	20, 20	Ongoing	NCT00317603, NCT00880464
Vaccination with a CD80-modified, devitalized breast cancer cell line (KS24.22)	Open label, phase 1 safety/efficacy study	Metastatic breast cancer	-	15	Completed	NCT01127074
Vaccination with four tumor-associated peptides	Open label, phase 1 safety/efficacy study	Triple negative breast cancer	Durvalumab	20	Recruiting	NCT02826434
Anti-TP53 TCR-gene engineered lymphocytes and autologous dendritic cell-adenovirus TP53 vaccine	Open label, phase 2 study	Progressive or recurrent metastatic breast cancer	Cyclophosphamide, fludarabine, aldesleukin (IL-2), filgrastim (G-	3	Completed	NCT00704938

			CSF)			
Treatment with a recombinant fusion protein of IL-2 linked to a single-chain TCR domain targeting TP53	Open label, phase 1 safety/efficacy study	Metastatic breast cancer	-	26	Completed	NCT00496860
Immunotherapy with modified TCR targeting NY-ESO-1 tumor antigen	Open label, phase 1/2 safety/efficacy study	NY-ESO-1 expressing breast cancer	Cyclophosphamide, fludarabine, aldesleukin (IL-2)	43, 36	Recruiting	NCT01967823, NCT02457650
Immunotherapy with modified TCR targeting CEA tumor antigen	Open label, phase 1 safety/efficacy study	Metastatic breast cancer, relapse or refractory breast cancer	-	60, 75	Ongoing/Recruiting	NCT01022138, NCT02349724
Engineered CAR T cells targeting mesothelin	Open label, phase 1 safety/efficacy study	Relapsed and refractory advanced triple negative breast cancer, metastatic HER2-negative breast cancer	Cyclophosphamide	20, 24	Recruiting	NCT02580747, NCT02792114
Engineered CAR T cells targeting CD133	Open label, phase 1 safety/efficacy study	Relapsed and refractory advanced triple negative breast cancer	-	20	Recruiting	NCT02541370
Engineered CAR T cells targeting EpCAM	Open label, phase 1 study	Recurrent breast cancer	-	30	Recruiting	NCT02915445
Engineered CAR T cells targeting ROR1	Open label, phase 1 safety study	Recurrent and metastatic breast cancer, triple negative breast cancer	-	60	Recruiting	NCT02706392
Engineered CAR T cells targeting HER2	Open label, phase 1/2 safety/efficacy study	Recurrent, metastatic or refractory HER2-positive breast cancer	-	60, 60	Recruiting	NCT02547961, NCT02713984
Engineered CAR T cells targeting MET proto-oncogene	Phase 1/2 safety/efficacy study	Operable triple negative breast cancer	-	15	Ongoing	NCT01837602
Vaccination with autologous DCs infected with an adenovirus expressing *HER-2*	Open label, phase 1 safety study	HER2-positive breast cancer	.-	5	Completed	NCT00197522
Vaccination with adenovirus-*TP53* transduced DCs	Open label, phase 1/2 safety/efficacy study	Recurrent and advanced breast cancer	1-methyl-d-tryptophan	44	Ongoing	NCT01042535
Vaccination with adenovirus-*HER2* transduced DCs	Open label, phase 1 safety study	HER2-positive breast cancer	-	65	Recruiting	NCT01730118

Vaccination with cyclin B1/WT-1/CEF pool-loaded DCs	Open label, phase 1/2 safety/efficacy study	Locally advanced, triple-negative breast cancer or ER-positive, HER2-negative breast cancer	Neoadjuvant chemotherapy	20	Recruiting	NCT02018458
Vaccination with autologous DCs pulsed with onco-peptides	Open label, phase 1/2 safety/efficacy study	Metastatic breast cancer	-	40	Completed	NCT00197925
Vaccination with DCs incorporating tumor blood vessel antigen-derived peptides	Open label, phase 1 safety study	Metastatic breast cancer	Gemcitabine	30	Recruiting	NCT02479230
Vaccination with DCs transfected with survivin, hTERT and TP53	Open label, phase 1 safety study	Metastatic breast cancer	-	31	Completed	NCT00978913

[a]Clinical Trial.gov Identifier

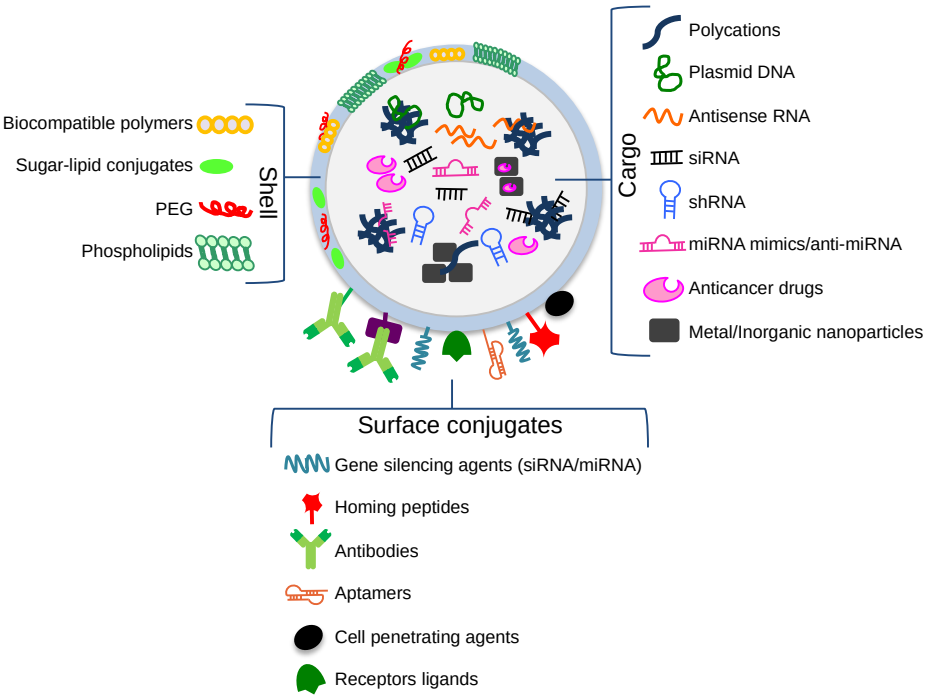
CD: Cytosine deaminase; CEA: Carcinoembryonic antigen; DC: dendritic cell; EpCAM: epithelial cell adhesion molecule; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HER2: erb-b2 receptor tyrosine kinase 2; HSV-1: herpes simplex virus type 1; hTERT: human telomerase reverse transcriptase; IL-2: Interleukin-2; IGFBP2: insulin-like growth factor binding protein-2; IGF1R: insulin like growth factor receptor-1; MAGEA4: Melanoma-associated antigen 4; MDR1: Multidrug resistance gene 1; MUC-1: Mucin-1; PRAME: Preferentially expressed antigen of melanoma; PSMA: prostate-specific membrane antigen; RRM2: Ribonucleotide reductase regulatory subunit M2; SSX: synovial sarcoma X; TCR: T cell receptor; TK: thymidine kinase; WT1: Wilms tumor protein 1; 5-FC: 5-fluorocytosine; 5-FU: 5-fluorouracil; 5T4: Tumor antigen 5T4.

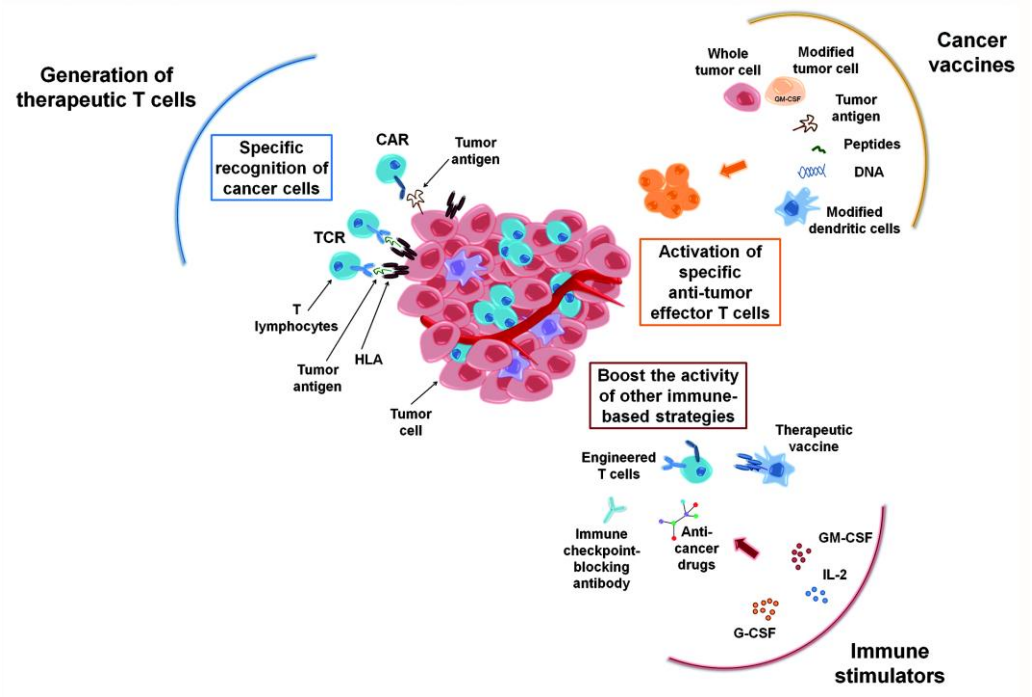
Figure legends

Figure 1. Schematic representation of multifunctional nanovectors for gene delivery. Multiple non-viral delivery systems have been developed for gene therapy. Different lipid formulations and functionalization strategies have been employed to improve the safety, stability and efficiency of nanovectors. The opportunity to load many types of cargos within the core, as well as to functionalize the outer layers, makes nanoparticles a versatile system for gene therapy.

Figure 2: Overview of the major immune-based approaches for cancer gene therapy. Autologous T cells can be genetically modified to express a novel TCR or a CAR to specifically recognize a tumor-associated antigen. Therapeutic cancer vaccines can derive from whole tumor cells, protein antigens, peptides, DNA, or dendritic cells, and are designed to potentiate anti-cancer immune response. These strategies can be administer in combination with immunostimulatory cytokines to boost anti-tumor activity.

CAR: Chimeric antigen receptor; HLA: human leucocyte antigen; IL-2: Interleukin-2; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TCR: T cell receptor.


Biocompatible polymers
Sugar-lipid conjugates
PEG
Phospholipids
Shell
Cargo
Polycations
Plasmid DNA
Antisense RNA
siRNA
shRNA
miRNA mimics/anti-miRNA
Anticancer drugs
Metal/Inorganic nanoparticles
Surface conjugates
Gene silencing agents (siRNA/miRNA)
Homing peptides
Antibodies
Aptamers
Cell penetrating agents
Receptors ligands


Generation of therapeutic T cells
Specific recognition of cancer cells
CAR
Tumor antigen
TCR
T lymphocytes
Tumor antigen
HLA
Tumor cell
Activation of specific anti-tumor effector T cells
Cancer vaccines
Whole tumor cell
Modified tumor cell
GM-CSF
Tumor antigen
Peptides
DNA
Modified dendritic cells
Boost the activity of other immune-based strategies
Engineered T cells
Therapeutic vaccine
Immune checkpoint-blocking antibody
Anti-cancer drugs
GM-CSF
IL-2
G-CSF
Immune stimulators