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Cancer Gene Therapy: Targeted Genomedicines

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1. Introduction

To date, traditional chemotherapy alone or in combination with immunotherapy and ionizing radiation modalities have been used to obliterate dividing aberrant cells in various tumors, while morbid statistics of cancer therapy show limited clinical successes.

Given the fact that malignant cells proliferate more rapidly than normal cells, damage to the cancer cells is anticipated to be markedly greater than normal cells. However, cancer cells generate chemoresistance mechanisms within the tumor microenvironment, while undesired toxicity may occur in the normal cells. For example, in colorectal cancer (CRC), there exist well-described sequences of mutational events that evince the shift of normal colon epithelium to premalignant adenoma and malignant adenocarcinoma. These events are 1) loss of the function of the adenomatous polyposis coli (APC) gene (encoding a protein involved in cell adhesion and transcription) in up to 85% of all cases of CRC, 2) mutation of KRAS (a GTP-ase that controls cell proliferation) in 50–60% of all cases of CRC, and 3) downregulated expression of the cell-adhesion transmembrane glycoprotein E-cadherin in almost 50–60% of all cases of CRC. Mutations in the mismatch-repair genes MLH1 and MSH2 contribute to genetic instability. Besides, there exist a number of genes alterations leading cells toward remodeling that include: 1) SMAD4 involved in the transforming growth factor signal transduction suppressing epithelial-cell growth, 2) INK4A involved in the retinoblastoma tumor-suppressor pathway, and 3) TP53 alterations increasing the resistance of cancer cells to apoptosis [1]. Similar molecular/cellular alterations occur in various solid tumors, highlighting the intricacy of biological events leading to initiation and progression of malignancies. Therefore, necessity for development and advancement of more effective modalities targeting such genetic changes is perceptible to achieve successful cancer treatment and cure. After decades of disappointment, targeted therapy of cancer has been advanced by integration of immunotherapy as well as gene and cell therapy. As proof-of-concept, recent clinical trials (e.g., anti-CTLA4 antibody, ipilimumab) have shown signifi-

cant increase in survival for patients with metastatic melanoma, for which conventional therapies have failed [2].

Targeted therapy of cancer using mAbs has provided great outcomes [3], while cancer gene therapy has not been as productive as immunotherapy from translational stand point. Efficient gene transfer strategy, as a fundamental step, continues to be the major determining factor for clinical successes of the gene therapy. In fact, there exist some hurdles that make gene therapy a formidable task. There are problems with delivery of sufficient copies of a gene (e.g., short interfering RNA (siRNA), antisense) to all tumor cells, whose biology appear to be very complex and ideally all the cancer-related genes must be controlled. Another barrier is the lack of proper gene delivery systems (GDSs) since the nonspecificity of GDSs makes gene therapy strategy somewhat uncertain. Overall, the current gene therapy approaches are capable of introducing genes into cells in vivo without discrimination within target and non-target cells. However, such unselective approach can impact both normal and aberrant cells. Incorporation of a homing device (e.g., monoclonal antibodies (mAb), antibody (Ab) fragments, or target specific aptamers) with an appropriate delivery system may result in cell-specific targeting and greater clinical outcomes [4].

The main gist of this chapter is to concisely provide information upon the specific gene therapy strategies and gene targets. We will discuss impacts of oncogenes, tumor suppressor genes and apoptosis-inducing genes on cancer gene therapy strategies as well as methods that specifically reactivate pathways that render the mutated cells susceptible to antitumor agents and immunotherapy. We will also remark on the cancer therapy opportunities through exploiting targeted nanogenomedicines.

2. Trajectory of gene therapy

Of many cancer therapy endeavors, cancer gene therapy has granted great hopes even though it is in its developmental trajectory. So far, more than 65% of the gene therapy trials have been devoted to the cancer diseases using various vectors (retrovirus (20%), advenovirus (18%), adeno-associated virusade (5%), lipofection (6%)) and naked/plasmid DNA (18.5%). Despite conducting more than 1186 cancer gene therapy trials (out of 1843), 45 have reached to phase III and only 1 is in phase IV [5]. At this stage, there exist 9 clinical trials of gene therapy that have been conditionally approved [4]. Most of these trials are conducted as adjuvant therapies, which clearly highlight needs for more effective gene therapy systems.

The foremost basis of gene therapy is to fix the genomic defects; nonetheless the gene therapy concept is going to be revolutionized by illumination of epigenomics and targeted genome-dicines. In tumor development, the origination of cancer is an intricate biological process, in which molecular changes at genomic/epigenomic levels play a central role. These molecular alterations can equip cancerous cells with unique molecular biostructures that play crucial roles in survival, progression and invasion of cancer cells. Such genomic/epigenomic altera-tions (e.g., changes in gene expression, mutations, gene deletion, DNA methylation/demethy-

lation and histone acetylation/deacetylation) have directed scientists to devise genomedicines to fix the genomic defects. It should be evoked that, unlike treatment strategies for genetic defects that need permanent expression of the corrected genes, cancer gene therapy is based on temporary and locally limited stimulation/suppression effects on desired gene(s). Further, malignant cells display specific gene markers that are different in nature or magnitude compared to the normal cells. These characteristics of cancer cells are deemed to provide a robust platform for specific targeted gene therapy that provides major advantages over current chemotherapy and immunotherapy modalities [6, 7].

Up until now, some domains of cancer gene therapy have been devoted greater attention, including: a) suppression of cancer cells by introducing genes into tumor cells to lead cells toward apoptosis (e.g., herpes simplex virus thymidine kinase, cytosine deaminase); b) inhibition of growth of cancer cells; c) enhancement of cancer cells chemosensitivity (p53, Bax); d) specific stimulation of the host's immune response against the cancer cells (tumor antigen, DNA vaccines, cytokine genes) by introducing the relevant genes into tumor cells or dendritic cells. Although use of genomedicines (e.g., antisense RNA, siRNA, ribozymes, DNAzyme and aptamers) have shown positive outcomes, their combination with other cancer therapy modalities including chemotherapy and immunotherapy can open other avenues for cancer therapy [8-10].

In addition, immune gene therapies (e.g., targeted DNA vaccine) exploit the lymphocytes and dendritic cells potentials, activating the immune system defense mechanisms against cancer cells. DNA vaccines possess intrinsic ability to activate multiple pathways of innate immunity, that also provide a unique opportunity to guide defined antigens, accompanied by specific activator molecules, through a patient's compromised immune system [11]. Further, suicide gene therapy tackles to deliver genes to the cancer cells, upon which cancer cells convert nontoxic prodrugs into active chemotherapeuties. In this approach, cancerous cells containing suicide genes are solely targeted through a systemic administration of prodrug. The suicide gene therapy is deemed to provide maximal inhibitory effects in cancer cells, but minimal toxic effects in normal cells [12]. Other than these strategies, antisense oligodeoxynucleotides (AS-ODNs) as a new class of molecularly targeted agents are in transitional trajectory from the laboratory into the clinic. A number of very important transcriptomic elements (e.g., VEGF, Ang-1, MDM2, protein kinase C-a, c-myb, integrin subunit b3, PKA-I, H-ras, bcl-2, c-raf, R1/R2 subunits of ribonucleotide reductase) have successfully targeted by AS-ODNs [13].

In contrast to AS-ODNs technology, the mechanism of silencing an endogenous gene through a homologous double-stranded RNA (dsRNA), which is termed post-transcriptional gene silencing (PTGS) or RNA interference (RNAi), is a natural mechanism by which mammalian cells can regulate expansion of genes. Accordingly, short interfering RNA (siRNA) can be used for gene silencing. It is currently the fastest growing sector for target validation and therapeutic [14]. Further, considering cancer cells scape from immune system within the tumor microenvironment, immune targeted gene therapy appears to provide an effective tactic for activation of the immune systems in such intricate microenvironment, whereby targeted gene therapy of angiogenesis and lymphangiogenesis bestow robust treatment possibilities [15].

3. Gene silencing as gene therapy modality

It is clear that the tumorigenesis results from clusters of several genetic and/or epigenetic events. Therefore, identification of the involved genes provides new targets toward effective treatment of malignancies. Of various gene therapy approaches, it is deemed that the silencing of cancer-causing genes can control the biological consequences at its genetic root and thereby cure the disease. Hence, development of agents capable of gene silencing is now considered as a rational strategy for cancer therapy, which can be accomplished by genomedicines. We will review gene silencing technologies in the following sections.

3.1. Antisense oligodeoxynucleotides for suppression of mRNA

During the last decade, we have witnessed emergence of synthetic AS-ODNs. They are primarily designed to attach selectively to the target transcriptomes and to disrupt the expression of a target gene. It should be evoked that the overall utility of AS-ODN as thera-peutic agent is dependent upon 1) the expression level of the target mRNA, 2) the optimal design of As-ODN, 3) the specificity of the AS-ODN to the target mRNA and 4) the availability of safe and highly efficient delivery systems. For example, we have shown that most of cationic polymers (CPs) and lipids used as GDSs to shuttle AS-ODNs specific to the epidermal growth factor receptor (EGFR) can also induce intrinsic cytotoxicity and toxicogenomics [16-22]. Previous studies have demonstrated that the viral vectors (e.g., adenovirus, adeno-associated virus (AAV), Epstein-Barr virus (EBV), herpes simplex virus type-1 (HSV-1), retrovirus, lentivirus, poxvirus, baculovirus) vectors can function as efficient vehicles for AS-ODN delivery. Of these, AAV vectors can be constructed to express short, distinct transcripts, a property that is useful for RNA - mediated inhibition of gene expression and successful delivery of the As-ODNs [23, 24]. Fig. 1 and Table 1 respectively represent the mechanism of action of AS-ODNs and their applications.
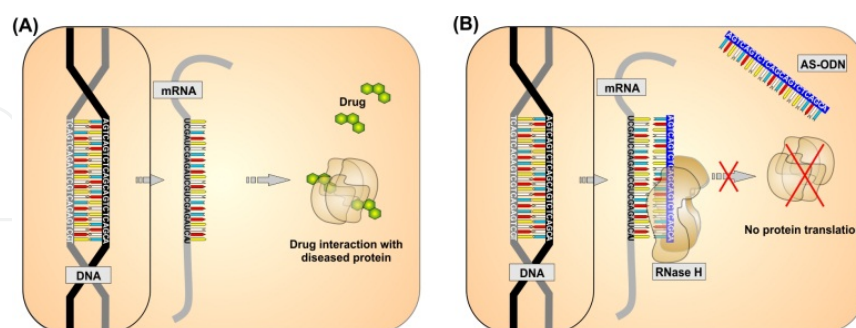
Figure 1. Mechanism of action of AS-ODN. A) Inhibition of proteins by small molecule drugs after translation. B) Suppression of mRNA by AS-ODN before translation in the presence of RNase H. This figure was adapted with permission from reference [4].

It is known that the oncogene E7 from high-risk human papillomavirus (HPV) strains has the potential to immortalize epithelial cells and increase cellular transformation in culture. Hence, to prevent the cervical cancer growth, the HPV16 E7 was inhibited by AS-ODN that was

delivered by a recombinant AAV (rAAV). It was found that such genomedicines can inhibit cell proliferation, induce apoptosis, reduce cell migration, and restrain in vivo proliferation of the cervical cancer CaSki cells [24]. Table 1 shows selected oncogenes targeted by AS-ODNs.

| Oncogene	Application	Ref
HER2 (c-erbB-2)	c-erbB-2 AS-ODN Inhibit serum-induced cell spreading of ovarian cancer cells	[27]
	Inhibitory effects of c-erbB-2 AS-ODN in uterine endometrial cancer Ishikawa cells	[28]
BCL-2	BCL-2 AS-ODN inhibits sensitize small cell lung cancer cells (in vitro and in vivo) to radiation	[29]
	Phase I/II study of G3139 (Bcl-2 AS-ODN) combined with doxorubicin and docetaxel in breast cancer	[30]
	Induction of apoptosis and increased chemosensitity in human prostate cancer cells by Bcl-2 AS-ODN	[31]
c-RAF-1	Phase I study of the c-raf-1 AS-ODN (ISIS 5132) combined with carboplatin and paclitaxel in patients with advanced non-small cell lung cancer	[32]
	Phase I study of the c-raf-1 AS-ODN (ISIS 5132) in patients with advanced cancer	[33]
c-FOS	Tissue-targeted antisense c-fos retroviral vector inhibits established breast cancer xenografts in nude mice	[34]
	c-fos AS-ODN control prostaglandin E2-induced upregulation of vascular endothelial growth factor in human liver cancer cells	[35]
c-MYC	c-myc AS-ODN sensitize human colorectal cancer cells to chemotherapeutic drugs	[36]
	inhibition of c-MYC by antisense phosphorodiamidate morpholino oligomer in prostate cancer murine models and humans	[37]

Table 1. Selected oncogenes targeted by AS-ODNs.

The effectiveness of the AS-ODNs have so far been shown in both target cells/tissue as in vitro models and animal in vivo models in particular for cancer therapy [13]. The mechanisms of action of the AS-ODNs seem to vary in different systems. In the case of hybridization and intramolecular and/or intermolecular interactions, their degradation pattern appears to be different. Many investigations have shown the anti-oncogenic impacts of AS-ODNs through targeting specific oncogenes. We have used AS-ODNs specific to EGFR and showed substantial inhibition of EGFR in A431 cells [25] as well as A549 lung cancer cells [26] using non-viral vectors as delivery system. The inhibitory impacts of AS-ODNs have been assessed through alterations in growth rate, morphology and molecular analysis. Various oncogenes have been targeted by AS-ODNs.

3.2. Small interfering RNA

The siRNA (also called as short interfering RNA or silencing RNA) is double-stranded RNA (dsRNA) molecules of 20-25 nucleotides. The siRNA gene-silencing mechanism is induced by

dsRNA and it is largely sequence-specific. RNA interference (RNAi) approach appears to be an extremely powerful tool for silencing gene expression in vitro [38]. Accordingly, huge researchers have been conducted to expand this technology toward in vivo applications [39]. Fig. 2 represents mechanism of siRNA in controlling the expression of a target mRNA.
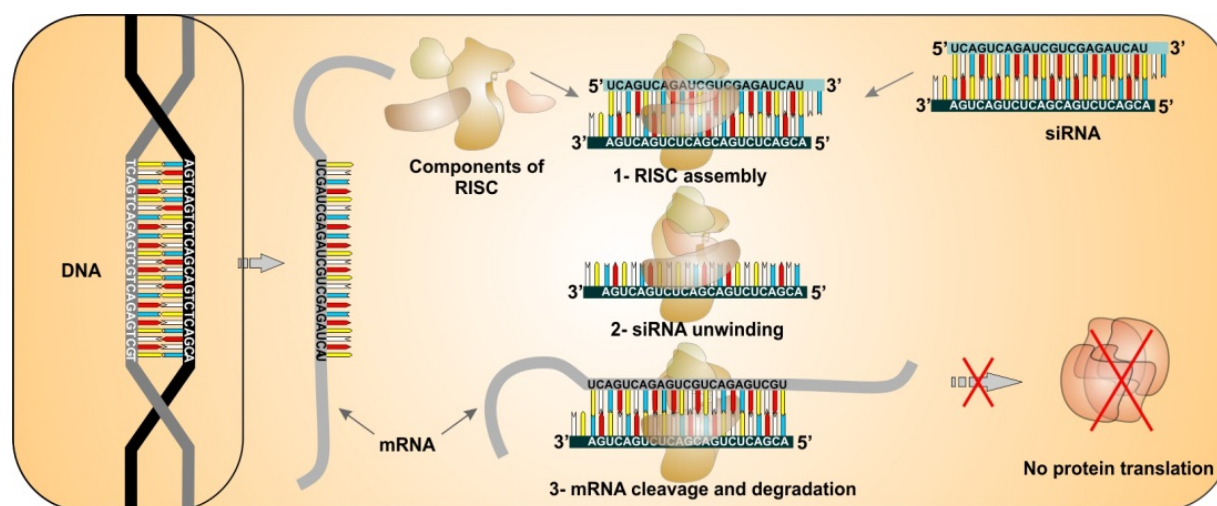
Figure 2. Cleavage and degradation of mRNA expression by siRNA. Short interfering RNAs (siRNAs) basically consist of two 21-25 single-stranded RNAs forming double stand RNA with overhangs at 3' end. The antisense strand of the siR-NA bound to RNA-induced silencing complex (RISC) can cleave the target mRNA. This figure was adapted with permission from reference [4].

Basically, investigation on RNAi has highlighted two distinct methodologies for gene silencing as: 1) cytoplasmic delivery of siRNA to target cells to imitate an endogenous RNAi mechanism and 2) nuclear delivery of gene expression cassettes expressing a short hairpin RNA (shRNA) that mimic the micro interfering RNA (miRNA) active intermediate of a different endogenous RNAi mechanism [40]. Both these approaches need safe delivery of gene materials into the target sites.

In fact, RNAi that was discovered initially in plants has been applied for various types of cancer as well as other diseases. Besides, RNAi technology seems to be the right tool for delineation of the functions and interactions of the thousands of human genes in high-throughput systems, which can also be harnessed in target validation technology. It is deemed that delivery of siRNAs as nanoformulations may resolve the inefficient delivery problem [41-43]. For example, a micelleplex system based on an amphiphilic and cationic triblock copolymer has been developed for delivery of siRNA specific to the acid ceramidase (AC) gene. In aqueous solution, the triblock copolymer (consisting of monomethoxy poly(ethylene glycol), poly(epsilon-caprolactone) and poly(2-aminoethyl ethylene phosphate)) can self assembles into positively charged (48 mV) micellar nanoparticles (MNPs) with an average diameter of 60 nm. Once exposed to siRNA, it can result in micelleplex that was shown to effectively internalize into the BT474 breast cancer cells and induce significant gene knockdown. Systemic delivery of micelleplex targeting AC gene was shown to significantly inhibit the tumor growth in a BT474 xenograft murine model without activation of the innate immune response [44].

Some chemotherapy agents are substrate of the efflux transporters (e.g., P-glycoprotein (P-gp), multidrug resistance proteins (MRPs)) that are often overexpressed on cancer cells developing resistance, while no safe inhibitor of P-gp is available. Simultaneous delivery of P-gp targeted siRNA and paclitaxel as poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles (NPs) decorated with biotin has been shown to overcome tumor drug resistance in both in vitro and in vivo [42].

3.3. Ribozymes and DNAzyme

After being discovered in early 1980s, ribozymes as a class of RNA showing catalytic activity to cleave RNA molecules in a sequence specific manner have been used for cancer therapy. They have been shown to perform excellent catalytic reactions with great precision, which can be encoded and transcribed from DNA. It was a decade later that DNAzymes have entered the scene of nucleic acid-mediated catalysis [45]. They are special class of nucleic acid chains, which usually consist of both double and single stranded regions that fold into a specific three-dimensional structure performing catalytic functions. Various ribozyme formats (e.g., hammerhead, hairpin, axhead, group I intron, and RNAse P) can be used as trans-acting catalysts. Of these, the hammerhead and hairpin ribozymes seem to be the most commonly used. For example, the efficacy of an anti-KRAS hammerhead ribozyme targeting GUU-mutated codon 12 of the KRAS gene was evaluated in a cell-free system and also in cultured pancreatic carcinoma cells [46]. Fig. 3 schematically exemplifies a morphology and cleavage mechanisms of Ribozyme (A) and DNAzyme (B).
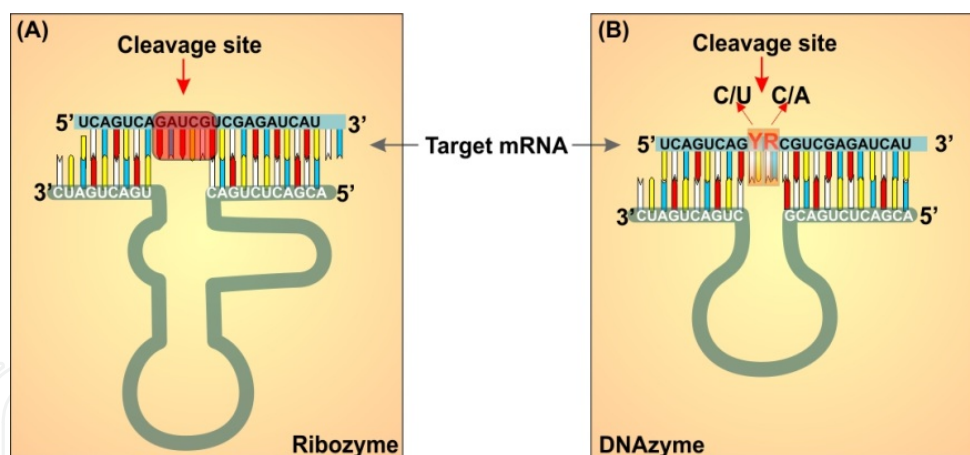
Figure 3. Schematic representation of morphology and cleavage mechanism of Ribozyme (A) and DNAzyme (B). This figure was adapted with permission from reference [4].

Tsuchida *et al.* showed that, in the cell-free system, the anti-KRAS ribozyme specifically cleaved KRAS RNA with GUU-mutation at codon 12. In the cell culture system, they showed that the anti-KRAS ribozyme significantly reduced KRAS mRNA level (GUU-mutated codon 12) in Capan-1 pancreatic carcinoma cells. Further, it has been proposed that trans-splicing ribozyme capable of specifically reprograming the human telomerase reverse transcriptase (hTERT) RNA can be harnessed as a useful tool for tumor-targeted gene therapy. Thus, a transcriptional targeting with the RNA replacement approach was implemented to target liver cancer cells

through combining a liver-selective promoter with an hTERT-mediated cancer-specific ribozyme [47]. To this end, Song *et al.* validated it in vivo by constructing an adenovirus encoding the hTERT-targeting trans-splicing ribozyme under the control of a liver-selective phosphoenolpyruvate carboxy kinase promoter. They found that intratumoral injection of this virus produced selective and efficient regression of tumors in mice [47].

It should be emphasized that the catalytic ribozyme core is basically attached to the specific regions of the target transcript through flanking antisense sequences. They have been designed to effectively cleave the targets transcript resulting in suppressed gene expression. For inhibition of gene expression, it is deemed that ribozymes are more effective than AS-ODNs because they cleave the target transcripts catalytically.

The DNAzyme (the so called deoxyribozyme) molecules consist of the 10-23 nucleotides, which bind to mRNA in a highly sequence-specific manner and cleave the RNA independent from RNase with the relatively stable chemistries used in oligodeoxynucleotide-based antisense reagents. The major obstacle in the further development of these technologies is a phenomenon that requires substantial development efforts invested in drugs of various classes, the uphill battle to affect cellular delivery in a targeted manner. This challenge is being met with a multidisciplinary approach with the hope that a greater understanding of each step of this process will enhance DNAzyme pharmacodynamics [45].

Owing to DNA backbone, DNAzymes have the advantage of being highly stable and cost-effective in compassion with RNAzymes and proteins. DNAzymes, similar to aptamers, can be isolated through a combinatorial in vitro selection process. Hence, they can be literally manipulated to meet the requirements and applied for engineering of targeted genoceuticals. Such characteristics make them excellent choice for dynamic control of nanomaterials assembly [48].

4. Target antigens and oncogenes

Tumor epithelial and endothelial cells as well as tumor associated cells represent unique marker molecules that can be harnessed for targeted therapy of cancer. For example, tumor vasculature varies significantly from its normal counterpart, representing unique cancer marker molecules. This has been emphasized through recent technologies including: immunohistochemistry laser-capture microdissection (immuno-LCM), genome-wide high-throughput screening, and proteomics. It is deemed that the vast array of vascular bed-specific markers may provide an exceptional platform for discovery of new therapeutics that target tumor microvasculature in various malignancies [49]. It is the same for tumor epithelial cells and tumor associated cells (TACs). Regarding the epithelial cells, EGFRs are the most studied cancer marker molecules (CMMs), whose upregulation in cancer cells was shown to be substantially down regulated with gene based medicines such as siRNA and AS-ODN. Likewise, vascular EGF and EGF-receptors have been shown to be upregulated in tumor endothelial cells and they can also be suppressed by genomedicines [50].

Malignant brain tumors (high-grade glioma), pancreatic cancer and malignant melanoma are among the most aggressive tumors known. Despite these facts, necessary translational steps are needed to be fulfilled for their clinical applications. For example, Antisense Pharma has recently taken an AS-ODN medication (Trabedersen or AP 12009) in several clinical trials. Trabedersen is a DNA-oligonucleotide that inhibits the synthesis of the cytokine transforming growth factor beta 2 (TGF-ß2) through specific binding to mRNA of TGF-ß2 that is overexpressed in many highly aggressive tumors suppressing the immune system activity [51-53].

4.1. Tumor antigen–specific vaccines and DNA vaccines

Cancerous cells of different types of tumors often display expression of aberrant genes such as: 1) mutated genes (e.g., mutated P53, RAS, BCR-ABL), 2) unique genes resultant from viral oncogenes (e.g., HPV E6 or E7), 3) overexpressed cancer specific genes (e.g., Her2, TGF-ß2, carcinoembryonic antigen, mucin).

These aberrant genes could be recognized by the host immune system, resulting in elimination of the cancerous cells expressing such oncogenes. However, cancer cells can circumvent from the anticancer activity of immune system within the permissive tumor microenvironment. Accordingly, the basis of the tumor antigen-specific vaccines is boosting the immune system harnessing these aberrant antigens. Nevertheless, success of this approach depends on identification and appropriate use of tumor specific genes [54-56]. In fact, vaccination against tumors may provide a selective destruction of malignant cells by the host's immune system, which can be applied as integrated system containing target gene(s) in recombinant vectors. Of viral vectors use in cancer vaccination, the recombinant AAV vectors appear to grant better clinical responses because of their low intrinsic immunogenicity, hence they have been employed to generate immune responses against specific antigens. For example, the safety of cytotoxic T lymphocytes (CTLs) infusion by transfected dendritic cells (DCs) with rAAV carrying carcinoembryonic antigen (CEA) cDNA was investigated in advanced cancer patients. For example, a total of 27 cancer patients with tumor tissue and/or sera-elevated level of CEA were treated with the rAAV-DC immunovaccine, which was well-tolerated showing no severe side effects in patients [57].

As the most potent antigen-presenting cells, DCs originate from the bone marrow and play a key role in the generation of immune responses. Further, peptide-based vaccination in cancer patients using DCs have resulted in promising outcomes. For example, to control the relapse and succumb to progressive disease in patients with advanced ovarian cancer, an immunotherapy approach was applied using CDs loaded with Her2/neu, hTERT, and PADRE peptides in a randomized open-label phase I/II trial. Despite showing modest immune responses, these peptide-loaded DC vaccination showed promising survival rate [58]. Fig. 4 represents the radar pattern of DNA vaccines in each phase of clinical trials.

It appears that we need to develop much more rational consolidative strategies for treatment of solid tumor in advanced stages since the applied strategies exploiting DCs (e.g., peptide pulsing with tumor antigens, transfection with DNA/RNA and transduction with tumor antigens encoding viral vectors) have not substantially generated antitumor immune responses.
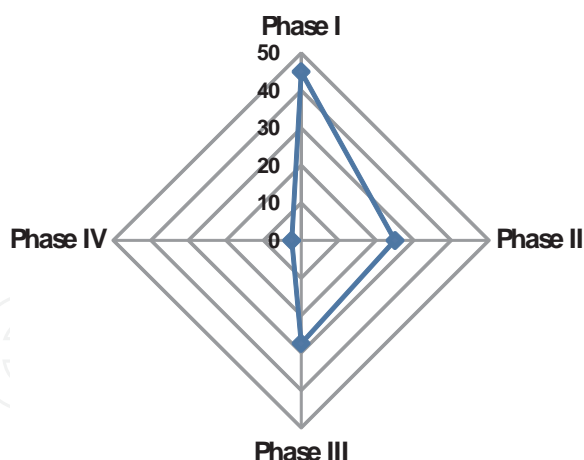
Figure 4. Selected DNA vaccines in clinical trials. Only 4 trials have reached to the phase IV trial, in which 3 of them are targeting human papillomavirus (HPV) and 1 targeting HPV and hepatitis B [59].

Ideally, for effective vaccination of any type of malignant disease, administered vaccine should activate both innate immunity and specific immune effector responses. Basically, the success of the vaccine therapy using immuno-stimulating genes depends on several parameters such as appropriateness of vector, suitable transgene design, inclusion/deletion of specific sequences, and optimization of necessary elements to induce secretion of the transgene product from the transduced cells. It also largely relies on the safe and efficient delivery of DNA into target cells. Development of the most current clinical trials has been based upon cytotoxic agents, immunotherapy and vaccination, while the mechanistic function of the DNA vaccines is different from these medicaments. They have several advantages over conventional vaccination modalities, including no risk of infection, antigen presentation by both MHC class I and class II molecules, polarizing T-cell helpers toward TH1/TH2 phenotypes, ease of preparation and cost-effectiveness [11, 60].

To date, over 730 DNA vaccines clinical trials have been undertaken. Of these, 156 are challenging different types of cancers [59]. A plasmid DNA encoding human tyrosinase (huTyr) has been approved by the US Department of Agriculture to treat canine melanoma [61]. The results supported the safety and efficacy of the huTyr DNA vaccine in dogs as adjunctive treatment for oral malignant melanoma. To date, no DNA vaccine has been approved by the U.S. Food and Drug Administration (FDA) for human, there exist more than 150 trials for different types of cancers. DNA-based vaccines have the advantage over conventional vaccines because they are able to induce both cell-mediated and humoral immunity, and to provide long-term responses with lower (in ng range) and fewer doses in a safer manner in comparison with conventional live vaccines. Further, they are cost-effective because of easier manufacturing process [56].

In 2010, sipuleucel-T (PROvenge®, Dendreon, USA) was approved by the FDA for treatment of asymptomatic/minimally symptomatic metastatic hormone-refractory prostate cancer (HRPC). PROvenge® is the first personalized medicine, which is a cellular immunotherapy agent and its administration demands 3 steps, as follow: 1) extraction of patient's antigen-presenting cells (APCs) through a leukapheresis procedure, 2) incubation with a fusion protein

PA2024 consisting of the antigen prostatic acid phosphatase (PAP) and an immune signaling factor granulocyte-macrophage colony stimulating factor (GM-CSF) that helps the APCs to mature, and 3) infusion of the activated blood product [62].

4.2. Tumor suppressor and apoptosis–inducing genes

It should be highlighted that the initiation of cancer is an intricate multi-cause process involving sequential activation of oncogenes and inactivation of tumor suppressor genes (TSGs) and apoptosis inducing genes (AIGs). Such genetic changes, subsequently, yield concomitant phenotypic alterations in the tumor cells resulting in cancer cells survival and progression. Thus, in addition to oncogenes, the tumor suppressor genes must be targeted by a designated genomedicine [63].

The pivotal roles of the TSGs and AIGs should be considered for cancer gene therapy, while little devotion has given to their biological impacts. The defects/mutated forms of these genes should be corrected through transfecting the normal forms which can be fulfilled through targeted systems; for more details on TSCs and AIGs, reader is directed to see reference [4].

4.3. Suicide gene therapy: A targeted genomedicine modality

Having harnessed suicide genes, a prodrug can be converted to a toxic metabolite. In fact, suicide gene therapy (SGT) is a unique approach that allows selective targeting through negative selection of malignant cells.

Using a designated prodrug, which can be activated only in aberrant cells producing the metabolizing enzyme, cancer cells can be specifically targeted by a nontoxic prodrug that metabolized into toxic metabolites. The herpes simplex virus thymidine kinase (HSV-TK) gene is the prototype gene, which can be transferred into tumor cells either by viral vectors or nonviral methods [64].

Suicide gene therapy using gene-directed enzyme/prodrug therapy (GEPT) was shown to improve the therapeutic efficacy of conventional cancer radiotherapy and chemotherapy without side-effects. Of the SGTs, the HSV- TK system gene therapy can sensitizes cells to the cytotoxic effects of designated drugs such as ganciclovir (GCV) and acyclovir (ACV). The HSV-TK-based SGT approach has resulted in promising outcomes in phase I/II study of glioblastoma, showing that brain injections of M11 retroviral vector-producing cells for glioblastoma HSV-1 TK gene therapy were well tolerated and associated with significant therapeutic responses [65]. Similar clinical outcomes have been reported for the treatment of melanoma [66]. In this study, although patients showed disease progression on long-term follow-up, retrovirus vector "M11"-mediated HSV-1 TK gene therapy was well tolerated over a wide dose range. Despite limited tumor response possibly due to poor gene transfer efficiency, necrosis following GCV administration in transduced tumors may indicate a potential for treatment efficacy. The HSV-TK based SGT has been reported as an effective system for treating experimental human pancreatic cancer [67].

In an interesting study, Aoi et al. capitalized on a physical method using ultrasound (US) and nano/microbubbles (NBs/MBs) to deliver exogenous genomedicines noninvasively into the

target cancer cells. They successfully harnessed a low-intensity pulsed ultrasound (1 MHz; 1.3 W/cm^2) and NBs/MBs to transduce the HSV-TK system using an in vitro model. They showed that addition of GCV to the transduced cells can lead to HSV-TK/GCV-dependent apoptosis [68].

Other paradigms of this approach are cytosine deaminase/5-fluorocytosine (CD/5-FC) and carboxyl esterase/irinotecan (CE/CPT-11). Further, genetically engineered stem cells (GES-TECs) have also been applied for GEPT [69]. While chemotherapy of brain tumors is often disrupted by the brain blood barrier (BBB) [70], GESTECs (consisting of neural stem cells (NSCs) expressing cytosine deaminase (CD) gene) have been employed as a novel cell therapy modality. The GESTECs were injected to xenograft mouse model of lung cancer metastasis to the brain produced through implanting the 549 lung cancer cells in the right hemisphere of the mouse brain. Two days after the injection of GESTECs, 5-fluorocytosine (5-FC) was administered via intraperitoneal injection. Histological analysis of extracted brain confirmed the therapeutic efficacy of GESTECs that converted the 5-FC into 5-fluorouracil resulting in the decreased density and aggressiveness of lung cancer cells [71].

Likewise, in a study, the GESTECs expressing either cytosine deaminase (CD) or carboxyl esterase (CE) showed profound inhibition of ovarian cancer cells SKOV-3 by converting prodrug 5-FC into 5-FU [72]. Table 2 represents the clinical trials for suicide gene therapy of cancer.

5. Immunogene therapy approaches for cancer

Cancer immunotherapy as an effective alternative treatment modality to chemotherapy arose from the notion that the immune system play a central role in prevention of the development/ progression of tumors, which is also called as immunosurveillance [73]. Perhaps the most compelling evidence for such tumor immunosurveillance is immune system activity in paraneoplastic diseases that are neurological disorders resultant from an anti-tumor immune response [74]. For progression, invasion and metastasis, a solid tumor must develop several critical abilities, including: 1) movement and migration potential, 2) capacity for degradation of extracellular matrix (ECM), 3) survival ability inside and outside of the tumor microenvironment escaping from immune system activity, and 4) propensity and quality of generation and progression in the new environment [75]. In fact, migrating malignant cells have capabilities to escape from immune system [76], invade and initiate a new life, perhaps through its pleiotrophic abilities activating a number of unique transcription factors, transporters and enzymes. Nevertheless, various solid tumors show some extend of immune system escape capabilities within the tumor microenvironment [76], where the anti-tumor immunity induced by T cells requires several mechanisms, including: a) recognition of an antigen by T cells receptors, b) co-stimulation by appropriate accessory molecules, and c) initiation of an inflammatory signal (the so called danger signal).

Inherently, based upon innate and adaptive responses of immune system, immunotherapy modalities are performed as "passive therapy" (using antibodies (Abs)/cytokines), "adaptive therapy" (in the form of the graft vs leukemia (GVL) reaction associated with the graft vs host

Clinical trial	US Trial ID	Malignancy	Intervention	Phase	Status
Randomized trial of suicide gene therapy and prostate cancer	NCT00583492	Prostate cancer	Biological: Ad5-yCD/ mutTKSR39rep-ADP; Radiation	II/III	Rg
Combining suicide gene therapy with chemoradiotherapy in the treatment of non-metastatic pancreatic adenocarcinoma	NCT00415454	Pancreatic cancer	Genetic: Ad5-yCD/ mutTKSR39rep-ADP	I	Td
Suicide gene therapy for donor lymphocytes infusion after allogeneic hematopoietic stem cell transplantation (ILD-TK01)	NCT01086735	Hematological malignancy	Biological: donor lymphocyte infusion	I/II	Rg
TK-based suicide gene therapy for hepatocellular carcinoma	NCT00844623	Carcinoma, hepatocellular	Genetic: TK99UN	I	Cd
An infectivity enhanced suicide gene expressing adenovirus for ovarian cancer in patients with recurrent ovarian and other selected gynecologic cancers	NCT00964756	Ovarian cancer	Genetic: Ad5.SSTR/ TK.RGD; Drug: GCV	I	Rg
CASPALLO: Allo-depleted T cells transduced with inducible caspase 9 suicide gene	NCT00710892	Lymphoblastic leukemia; lymphoma	Biological: Allodepleted T Cells	I	Active
Administration of donor T cells with the caspase-9 suicide gene	NCT01494103	Various types of leukemia; non-Hodgkin's lymphoma	Biological: Allodepleted T cells transduced with caspase 9; Drug: AP1903	I	Rg
Infusion of donor lymphocytes transduced with the suicide gene HSV-TK in patients with haematological malignancies	NCT00423124	Hematological malignancies	Genetic: HSV-TK	I/II	Active

Table 2. Clinical trials for suicide gene therapy of cancer. Ad5-yCD/mutTKSR39rep-ADP: Replication-competent adenovirus; Ad5: Adenovirus; yCD: Yeast cytosine deaminase; ADP: Adenovirus death protein; Td: terminated; Rg: recruiting; Cd: completed; IMRT: Intensity-modulated radiation therapy; TK99UN: An adenoviral vector containing herpes simplex virus's thymidine Kinase; GCV: Ganciclovir; AP1903: a lipid permeable, synthetic organic compound used exclusively in conjunction with a chemical inducers of dimerization (CID) therapy; HSV-TK: herpes simplex virus's thymidine Kinase. Data were adapted with permission from reference [4].

(GVH) reaction) or "active therapy" by stimulating the immune system [77]. Basically, autologous antigen-specific T cells can be expanded ex vivo and then re-infused into patients to boost T cells-based immune system activities. DCs, which play a central role in immune

system activities due to their ability to control both immune tolerance and immunity, have been extensively used as a cell-based immunotherapy modality [74]. While tumor cells themselves are poor antigen-presenting cells (APCs), DCs are potent APCs. Fundamentally, the aim of DCs based immunotherapy is to elicit tumor-specific effector T cells (CD4+ T cells, CD8+ T cells and B cells) that can effectively reduce the tumor mass and can also induce immunological memory to control tumor relapse [74]. The first step of DCs-based vaccination is to provide DCs with tumor-specific antigens, which can be performed through ex vivo cultivation of the patients-derived DCs with an adjuvant for DC maturation and the tumor-specific antigen. The processed DCs can be then injected back into the patient. Alternatively, DCs can be induced to take up the tumor-specific antigen in vivo [74]. This approach has been harnessed as vaccination modality in various cancers. For example, phase I/II randomized trial of DCs-based vaccination with or without cyclophosphamide have recently been conducted for consolidation therapy of advanced ovarian cancer in first or second remission [58]. It was shown that the peptide-loaded DC vaccination induced modest immune responses, while the survival rate was promising [58].

In a study, Coukos' group has investigated the mechanism underlying cooperation between oncolytic HSV and host effector immune mechanisms in a syngeneic murine model of ovarian carcinoma. They showed that therapeutic administration of HSV-1716 (a replication-restricted mutant) can result in significant reduction of tumor growth and improved survival rate. Intratumoral injection of HSV-1716 elicited expression of some key elements (IFN-γ, MIG, and IP-10) and significant increase in the number of tumor-associated natural killer (NK) and CD8+ T cells. Ascites from HSV-1716-treated animals efficiently induced in vitro migration of NK and CD8+ T cells that was dependent upon the presence of MIG and IP-10, in which monocytes and DCs appeared to be responsible for the production of MIG and IP-10 [78]. This study clearly indicate that, in ovarian carcinoma, monocyte-derived DCs produced large amounts of IFN-gamma and upregulated MIG and IP-10 expression upon HSV-1716 infection, which may favor antitumor immune response upon oncolytic therapy.

Thus far, CDs-based vaccination has been harnessed in over 150 clinical trials [59]. Nevertheless, the overall results obtained from the human clinical trials capitalizing on DCs have shown promising clinical outcomes resulting in significant induction of clinically meaningful antitumor immunity even with no apparent side effects or toxicities. This modality is a perfect paradigm for personalized medicines.

6. Anti–angiogenesis gene therapy

Several critical steps are involved during angiogenesis, including: proliferation of the endothelial cells (ECs), migration of the ECs, degradation of the basement membrane, and formation of the new lumen organization. Such biological event is controlled by proangiogenesis and antiangiogenesis factors liberated by various cells (activated ECs, monocytes, smooth muscle cells, pericytes and platelets) into the blood circulation [79]. Tumors need angiogenesis for survival and growth, thus inhibition of angiogenesis can be an effective strategy for cancer therapy.

Administration of endogenous inhibitors of angiogenesis are associated with some hindrances (e.g., high dose requirements and some instability of the corresponding recombinant proteins), hence gene therapy of angiogenesis may be an effective approach to battle malignancies. It should be noted that tumors secrete a number of "angiogenesis" factors, whose encoding genes can be targeted.

Selected angiogenesis factors include: vascular endothelial growth factor (VEGF), thrombospondin-1 (THBS1), endostatin, tumstatin, arresten, canstatin, vastatin, restin, angiostatin, 16 kD human prolactin fragment (16K hPRL), platelet factor-4 (PF4), interferon-inducible protein-10 (IIP10), angiopoietins, interleukin-12 (IL-12), interleukin-18 (IL-18), interferons (IFNs), endothelial-monocyte activating polypeptide-II (EMAP-II), tissue Inhibitors of metalloproteinases (TIMPs), tumor necrosis factor-α (TNF-α, transforming growth factor (TGF), pleiotropin, fibroblast growth factor (FGF), placental growth factor (PGF), and platelet derived endothelial cell growth factor (PD-ECGF) [79]. Of these, VEGF is the most studied target. It carries out multifaceted functions in tumor development, in which several isoforms impose distinct biologic functions and clinical implications. Several strategies have been carried out to control VEGF with some successes. Coukos' group has successfully used DNA vector-based RNA interference (RNAi) by inserting RNAi sequences targeting murine VEGF isoforms in downstream of an RNA polymerase III (Pol III) promoter, which may have potential applications in isoform-specific "knock-down" of VEGF. They compared two Pol III promoters, U6 and H1, in their efficiency forsiRNA expression. Large molecular weight VEGF isoforms were specifically reduced in vitro in the presence of isoform-specific RNAi constructs. Additionally, H1 promoter may be superior to U6 promoter when used for vector-based RNAi of VEGF isoforms. They proposed this novel strategy as an effective tool to investigate the functionalities of various VEGF isoforms and also concluded that, to develop such novel RNAi strategy as a practical research tool and feasible cancer therapy approach, identifying the most efficient targeting sequence and developing an efficient delivery system are vital steps [80].

The effect of INF-β gene therapy on the growth of human prostate cancer was determined in nude mice bearing PC3MM2 cells. It was found that the intralesional delivery of adenoviaral vector encoding murine IFN-β was able to suppress the growth of tumor in a dose-dependent manner, perhaps through induction of INF-β and inducible nitric oxide synthase (iNOS) as well as reduction of basic FGF and TGF-β1 resulting in inhibition of angiogenesis [81].

In a study, rAAV vectors were constructed to express endostatin (rAAV-endostatin) or the antiangiogenic domain of thrombospondin-1 3TSR (rAAV-3TSR) and applied to a mouse angiogenesis model. The rAAV-mediated gene delivery resulted in inhibition of VEGF-induced angiogenesis, in which pretreatment of mice with i.m. or intrasplenic injection of rAAV-endostatin or rAAV-3TSR significantly inhibited tumor growth [82].

The rAAV vectors carrying IL-12 and angiostatin-like molecule (K1-3) were administered to a subcutaneous hepatoma model in mice (Hepa129 cells in C3H mice). It was found that injection of rAAV-K1-3 or rAAV-IL-12 into tumor nodules resulted in a significant dose-dependent reduction in tumor growth, while the survival rate was significantly improved in the IL-12 treated mice, but not in the K1-3 treated mice. Combined therapy of these genomedicines, however, did not further improve antitumor efficacy compared with the monotherapy [83]. Table 3 represents selected examples for angiogenic gene therapy trials.

| Clinical trial	US Trial ID	Malignancy	Intervention	Phase	Status
Phase I - Pre-Radical Prostatectomy RTVP-1 Gene Therapy for Prostate Cancer	NCT00403221	Prostate Cancer	Genetic: RTVP-1 Gene	I	Cd
Trial of E10A in Head and Neck Cancer	NCT00634595	Head and neck squamous carcinoma; Nasopharyngeal carcinoma	Drug: E10A, Cisplatin, Paclitaxel	II	NA
Safety and Efficacy of Adenoviral Endostatin in the Treatment of Advanced Solid Tumor	NCT00262327	Advanced solid tumor	Drug: Antangiogenesis; Genetic: endostatin gene	I	NA
Gene Therapy in Treating Patients With Unresectable, Recurrent, or Refractory Head and Neck Cancer	NCT00004070	Head and neck cancer	Biological: interleukin-12 gene	I/II	NA
Interleukin-12 Gene Therapy in Treating Patients With Skin Metastases	NCT00028652	Metastatic cancers	Biological: interleukin-12 gene	I	Td
Interleukin-12 Gene and in Vivo Electroporation-Mediated Plasmid DNA Vaccine Therapy in Treating Patients With Merkel Cell Cancer	NCT01440816	Skin cancers	Biological: interleukin-12 gene; electroporation-mediated plasmid DNA vaccine therapy	II	Rg
Treatment of B-CLL With Autologous IL2 and CD40 Ligand-Expressing Tumor Cells + Lenalidomide	NCT01604031	Chronic lymphocytic leukemia	Biological: B-CLL Vaccine; Drug: Lenalidomide	I/II	NA

Table 3. Selected paradigms for angiogenic gene therapy trials. RTVP-1: related to testes-specific, vespid, and pathogenesis protein; Cd: completed; NA: not available; E10A: an adenovirus carrying human endostatin gene [84]; Td: terminated; Rg: recruiting. Data were adapted with permission from reference [4].

7. Targeted nanogenomedicines: Nanotechnology and gene therapy integration

Integration of nanotechnology with gene therapy has resulted in production of advanced nano-scaled genomedicines that can be armed with homing devices to deliver the gene-based cargos to the target sites through both passive and active targeting mechanisms. It should be high-lighted that the lipids or polymers used for formulation should be positively charged to be able to condense the negatively charged nucleic acids. However, we have shown that both

cationic lipids (CLs) and polymers may induce inadvertent intrinsic gene expression, masking/stimulating some undesired gene activities [17, 19-21, 85, 86]. Fig. 5 represents schematic structure of the advanced nanoformulations for genomedicines.
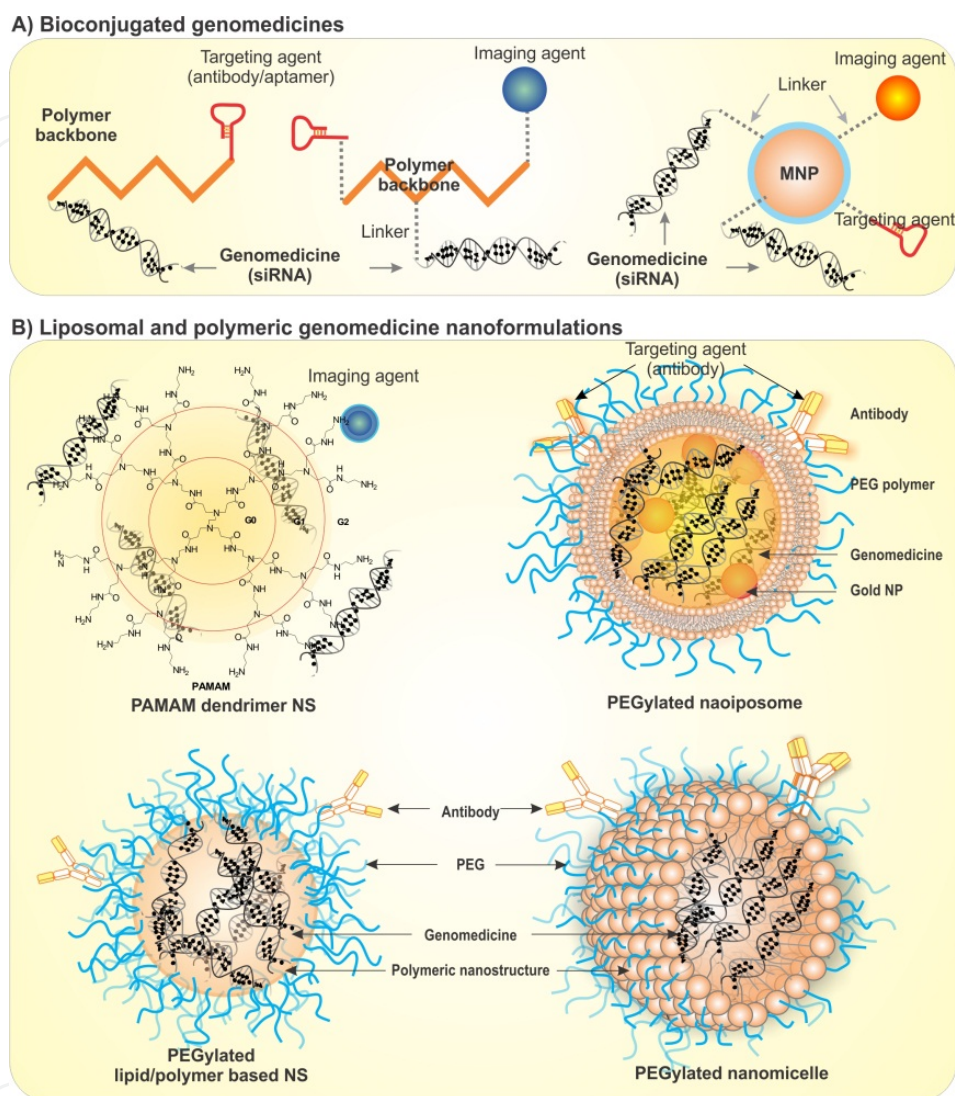
Figure 5. Schematic structures of advanced nanogenomedicines. A) Bioconjugations of genes with polymeric back-bone and grafted homing and imaging moieties. B) Polymeric and liposomal gene containing nanoformulations. This figure was adapted with permission from reference [4].

7.1. Bioconjugation and PEGylation

Lipids and polymers, based upon their end groups, can be conjugated with different moieties such as imaging devices (fluorescent dyes, quantum dots) and homing devices (antibody, peptide, aptamer). Post-formulation conjugation of NPs are basically performed through chemical grafting using homobifunctional crosslinkers (*e.g.*, N-hydroxysuccinimide (NHS) esters, immidoesters, sulfhydryl-reactive crosslinkers, hydrazides) or heterobifunctional crosslinkers (*e.g.*, sulfhydryl-reactive and photoreactive crosslinkers like N-succinimidyl-3-(2-

pyridyldithio)propionate (SPDP), LC-SPDP, and Sulfo-LC-SPDP) [87]. Decoration with homing devices can arm them to target cancer cells and deliver the gene-based cargo directly to the tumor microenvironment and thereby cancer cells, but not normal cells/tissues. Antibodies can be modified via amine groups using 2-iminothiolane (Traut's reagent) and conjugated to NPs. They can also be activated with, N-succinimidyl S-acetylthioacetate (SATA) or SPDP, in which the active NHS ester end of SATA or SPDP can react with amino groups in proteins and other molecules to form a stable amide linkage. Further, conjugation of the NPs with PEG (the so called PEGylation) can favor the pharmacokinetics of these NPs prolonging the circulation period that grant a proper time frame for NPs' accumulation in the tumor microenvironment. Although attaching poly ethylene glycol (PEG) (i.e., PEGylation) is the most effective method to reduce protein immunogenicity and to avoid the RES system clearance, several other polymers have successfully been implemented as alternative to PEG, including poloxamer, polyvinyl alcohol, poly(amino acid)s, and polysaccharide. However, PEG is still the most widely used polymer to engineer stealth NPs [88]. For nanoliposomes, PEG-lipid (such as PEG-DSPE) is usually inserted into liposomes to form a hydrated layer on the liposome surface.

7.2. Bioimpacts of nanogenomedicines

Typically, tumor microvasculature display discontinuous fenestrated morphology characteristics with gaps and pores between endothelial cells, in which the pore sizes are at a range of 100 nm to 1000 nm [89]. For instance, subcutaneously grown tumors were reported to have profound fenestration, showing pore sizes at a range of 200 nm to 1200 nm [90]. Most tissues present tight junctions between cells with intercellular openings smaller than 2 nm and around 6 nm in post-capillary venules, and tissues with discontinuous fenestrated endothelium such as kidney glomerulus and sinusoidal endothelium of liver have larger junctions with pore sizes of 40-60 nm and 70-150 nm, respectively [91]. As a result, NPs with size ranging 150-250 nm can substantially extravasate showing significant enhanced permeation and retention (EPR) effects within the tumor microenvironment [92]. Since long circulation of NPs in blood is a pivotal requirement for their successful *in vivo* applications, they are basically grafted with PEG that provide greater hydrophilicity and longer circulation in blood resulting in greater accumulation within the tumor microenvironment [93]. The naked gene based medicines such as AS-ODN and siRNA can be simply degraded and destroyed by the nuclease enzymes within blood, thereby not being taken up by the target cells and even giving a rise to undesired harmful immune reactions. Thus, nano-scaled protected gene medicines will provide desired canonical outcomes. Recently, it was shown that the siRNA protected by cyclodextrin-containing polymers (the basis of the RONDEL platform) can literally get to the proposed target site and impose the intended impacts [94].

7.3. Liposomal NPs

Nanoliposomes are basically formulated using solvent evaporation method to make lipid film that is then subjected to hydration. In the presence of surfactant, the hydrated lipids form multilamellar vesicles (MLVs) and unilamellar vesicles (ULVs) with a diverse size, ranging

from μm to nm, respectively. Sonication, homogenization and extrusion are the methods used for preparation of liposomes. In practice, based on end-point aims, different compositions of lipids can be used to engineer the intended liposomes. Mainly, lipophilic compounds (*e.g.*, phosphatidylcholine (PC), cholesterol (Chol), designated amounts of functionalized lipids and lipophilic drugs) are dissolved in a solvent (*e.g.*, chloroform or 3:1 ratio of chloroform: methanol). To form lipid film, the solvent is then evaporated using a rotary evaporator at 40-60 °C. The lipid film is rehydrated with stirring (~250 rpm) for 1 hr in the presence of surfactant such as Tween 20/Tween 80 under pulsed sonication (20 s ON, 10 s OFF intervals to avoid over-heating) for 10 min. As an alternative approach, the mixture can be homogenized by a high-speed homogenizer at 16,000 rpm for 5 min. To make uniform nanoliposomes, they can be extruded through polycarbonate filters with a designated pore size of (*e.g.*, 200 nm or 100 nm) for several times. The nanoliposomes can be then lyophilized for future use, reader is directed to see [95]. Mixture of polycationic lipids with plasmid DNAs can form self-assembled liposomal structures. To this end, several lipids have been exploited [96, 97], including: mixture of dioleoyl phosphatidylethanolamine (DOPE); 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA); N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethy- lammonium trifluoroacetate (DOSPA); N,N-distearyl-N,N-dimethylammonium bromide (DDAB); dioctadecylamidoglycyl carboxyspermine (DOGS); N-(2,3-dioley-loxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP); N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE); dioleyl-N,N-dimethylammonium chloride (DODAC); dipalmitoylphosphatidylcholine (DPPC); and 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol). However, most of these cationic lipids induce nonspecific gene expressions [17, 20, 85].

CLs are able to yield relatively high transfection efficiency in vitro and in vivo, and accordingly they have been progressed toward clinical trials. Precise advantages of this approach are 1) the simplicity of the DNA/liposome formulation as lipoplex, 2) the stability of the formulation and gene components protection, and 3) the robustness and applicability of the method for delivery to different types of solid tumors. For example, using cationic lipids, 16K hPRL was formulated as cationic liposomes and administered subcutaneously to a B16F10 mouse melanoma model. The results revealed that administration of the liposomal formulation of 16K hPRL gene can effectively maintain antiangiogenic activities in mice [98]. Table 4 represents selected gene therapy trials using liposomal formulations.

In another study, to monitor breast cancer processes, a unique liposomal formulation was applied as quantitative bioluminescence imaging (BLI) method. A breast cancer model was created by injection of 4T1 cells carrying a reporter system encoding a double fusion reporter gene consisting of firefly luciferase (Fluc) and green fluorescent protein (GFP) into BALB/c mice. Nanoliposomes loaded with a triple fusion gene containing HSV-TK and renilla luciferase (Rluc) and red fluorescent protein (RFP) were administered, and subsequently mice were treated with GCV. This approach resulted in monitoring of the tumor growth by BLI, while the treatment delivery of nanoliposomes was efficiently tracked by Rluc imaging [99]. Further, to avoid rapid clearance by RES after an intravenous injection, stealth PEGylated liposomes have resulted increased serum half-life and greater EPR. These stealth nanolipo-

| Clinical trial	US Trial ID	Malignancy	Intervention	Phase	Status
Gene Therapy in Treating Patients With Advanced Head and Neck Cancer	NCT00009841	Advanced head and neck cancer	Liposomal formulation of EGFR antisense	I	NA
FUS1-nanoparticles and Erlotinib in Stage IV Lung Cancer	NCT01455389	Lung cancer	DOTAP:Chol-fus1; Erlotinib; Dexamethasone	I/II	Active
Study to Determine the Maximum Tolerated Dose of LErafAON in Patients With Advanced Solid Tumors	NCT00024661	Advanced solid tumors	LErafAON	I	Cd
EphA2 Gene Targeting Using Neutral Liposomal Small Interfering RNA Delivery	NCT01591356	Advanced solid tumors	siRNA-EphA2-DOPC liposomes	I	NA
C-VISA BikDD: Liposome in Advanced Pancreatic Cancer	NCT00968604	Advanced pancreatic cancer	BikDD Nanoparticles	I	Active

Table 4. Gene therapy clinical trials using liposomal formulations. EGFR: epidermal growth factor receptor; NA: not available; Cd: completed; LErafAON: liposomes carrying antisense oligonucleotide against the Raf-1 protein; siRNA: small interfering RNA; EphA2: ephrin type-A receptor 2; C-VISA BikDD: liposome consists of a pancreatic-cancer-specific expression vector "VISA" (VP16-GAL4-WPRE integrated systemic amplifier) and a pancreatic-cancer-specific promoter CCKAR (cholecystokinin type A receptor) (CCKAR-VISA or C-VISA) which drives expression of the gene BikDD, a mutant form of the potent proapoptotic gene Bik (Bcl-2 interacting killer). Data were adapted with permission from reference [4].

somes can be further decorated with homing devices (Ab, ligand) and imaging devices to develop targeted stealth nanoliposome for safe i.v. delivery of gene based medicines [100]. For example, transferrin (Trf) receptor-targeted liposomes (Trf-liposomes) encapsulating anti-BCR-ABL genomedicine (siRNA or AS-ODN) has resulted in significant delivery of cargo genes in chronic myeloid leukemia [101]. Folate receptor-targeted liposomes have also been used as cancer specific vectors [102]. For efficient delivery of siRNA to neuroblastoma (the most common solid tumor in early childhood), Adrian et al. engineered liposomal nanoformulation (190 to 240 nm) containing siRNA armed with anti-Disialoganglioside (GD2) Ab for selective interaction with neuroblastoma cells [103]. They showed a significant association of liposomes with neuroblastoma cells and effective delivery of siRNA with anti-GD2 Ab-armed liposomes.

7.4. Polymeric gene delivery nanosystems

To engineer polymeric NPs, various synthetic and biodegradable polymers have so far been exploited. Of these, biodegradable polymers provide better clinical outcomes. Among biodegradable polymers, poly(lactic-co-glycolic acid) (PLGA) as a copolymer approved by FDA is the most widely used polymer. For engineering PLGA NPs, depending on physico-

chemical property of drug and desired emulsion (single or double), solvent evaporation or solvent diffusion methods are mainly recruited [104]. We have developed folate receptor targeting PEGylated PLGA NPs for delivery of nucleic acids (Fig. 6).
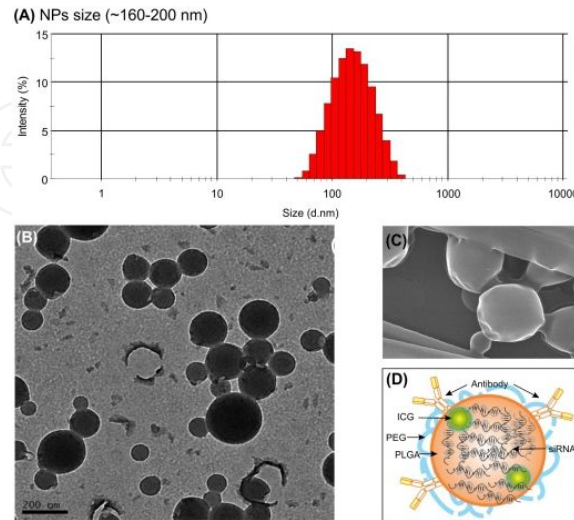
Figure 6. Size and morphology of PLGA nanoparticles. A) Dynamic light scattering (DLC) analysis of PEGylated PLGA NPs encapsulating siRNA. B) Transmission electron microscopy (TEM) micrograph of PLGA NPs. C) Scanning electron microscopy (SEM) micrograph of clusters of PLGA NPs. D) Schematic presentation of antibody armed PEGylated PLGA nanoparticle (our unpublished data).

Self-assembled micellar nanoformulations are another type of NPs that are widely used as gene delivery nanosystems, in which the positively charged polymers can interact with the negatively charged nucleic acids forming nanomicelles under sonication. Cationic polymers (e.g., linear and branched polyethyleneimine (l-PEI and b-PEI), poly(L-lysine), and polyamidoamine (PAMAM) dendrimers) can condense the nucleic acids forming polyplexes as either uni-molecular or multi-molecular complexes. Unfortunately, similar to CLs, cationic polymers can also induce intrinsic toxicogenomics [86] and cytotoxicity [16]. To achieve efficient target-specific gene transfer, these polymers can covalently be modified by conjugating targeting ligands. The ligand-armed systems can then target the cells that present the specific cellular receptors. Because of their nano-scaled size (100-200 nm in diameter), they can be taken up by cells through receptor-mediated endocytosis. For example, a Trf-modified cyclodextrin polymer-based gene delivery nanosystem has been developed. These Trf-armed PEGylated cyclodextrin show increased stability in biological fluids and active targeting potential via transferrin, retaining high binding affinity toward Trf receptor and profound transfection of the target leukemia cells (K562 cells) through both passive and active targeting [105]. Recently, Hatefi's group engineered a novel multi-domain biopolymer, consisting of: 1) repeating units of arginine and histidine to condense pDNA and lyse endosome membranes, 2) a HER2 affibody as targeting moety, 3) a pH responsive fusogenic peptide to destabilize endosome membranes and enhance endosomolytic activity of histidine residues, and 4) a nuclear localization signal to enhance translocation of pDNA towards the cell nucleus [106]. They showed that pDNA was condensed into biopolymeric Nps and protected from serum endo-

nucleases, while targeting HER2 positive cancer cells, and metabolized by endogenous furin enzymes to reduce potential toxicity. Later on, synthesis of a targeted PEI polymer was reported, in which the PEI polymer was conjugated to angiogenic vessel-homing peptide Ala-Pro-Arg-Pro-Gly (APRPG) through PEG spacer [107]. The PEI-PEG-APRPG was shown to effectively condense siRNA into 20-50 nm NPs that can substantially impose inhibitory effects in vitro with profound EPR in vivo targeting tumor vasculature through VEGF.

8. Tissue specific gene therapy

In addition to targeted nanomedicine gene therapies, tissue specific gene therapy provides a robust targeted approach. We describe some of the cell/tissue specific applications in the following sections.

8.1. Cancer Stem Cells (CSCs)

Some of the transit-amplifying cells in the cancer population appear to remain immature within the differentiating cells. These classes of cells that act as cancer cells progenitor are called cancer stem cells, which are deemed to be one of the reasons for cancer relapse that are hardly responsive for treatment. The poor prognosis and responsiveness of patients with relapsed aggressive metastatic tumors necessitate the development of more effective tumor-selective therapies towards cells that cause such relapse, while the conventional therapy target the differentiated cancer cells. Therefore, to be maximally effective, gene therapy of cancer should target both the resting stem cells and the proliferating cells of the cancer [108]. To this end, several translational approaches have been undertaken to target CSCs, including use of oncolytic viruses that may offer an effective way to specifically target and eradicate CSCs. Of these, conditionally replicative adenoviruses (CRAd) are considered as promising virotherapy systems [109]. Considering the plasticity of CSCs, apoptosis-inducing strategy can be used to eliminate these cells by harnessing genes such as TRAIL, BCL-2 family and XIAP as targeted therapies [110].

8.2. Mesenchymal Stem Cells (MSCs) as a gene therapy carrier

To date, as a personalized medicine, cell-based therapy of cancer has been considered as a promising modality. Of these, MSCs seem to hold great potential as targeted-delivery vehicle in cancer gene therapy [111]. Their propagation in culture is simple, also shows contingency toward genetic modification in order to express therapeutic proteins. Above all, MSCs possess inherent tumor-tropic and migratory properties that allow them to serve as robust cell based carrier as targeted drug delivery systems for isolated tumors and metastatic diseases [112]. In a study, the migration ability of MSCs toward prostate cancer cells (in vitro and in vivo) and incorporating into the tumor mass was investigated. The infected cells with HSV-TK gene were shown to maintain their tumor tropism capabilities and significantly inhibited the growth of subcutaneous PC3 prostate cancer xenografts in nude mice in the presence of GCV [113]. Similar strategy was applied to evaluate the impact of the suicide gene therapy by MSCs in

normal cells in brain using a rat model. It was found that the tumoricidal bystander effect in the HSV-TK gene therapy using MSCs and GCV does not injure normal brain tissues [114]. In another study, umbilical cord blood MSCs were used to deliver the transgenic LIGHT (TNFSF14) to the target tumor cells in vivo. The transfected MSCs with lentiviral vectors carrying LIGHT genes demonstrated a strong suppressive effect on tumor growth, in which pathological sections of the tumor tissues showed significant induction of apoptosis and occurrence of tumor necrosis in tumor cells [115]. The potential of genetically modified MSCs expressing IFN- β was assessed in an immuno-competent mouse model of prostate cancer lung metastasis. Significant reduction in tumor volume in lungs was seen following IFN- β expressing MSC therapy, perhaps through induction of apoptosis and increase in the natural kill cell activity [116]. The MSC-based gene therapy is still in its infancy era and need much more investigation prior to its clinical applications even though the MSCs themselves are under clinical trials [117].

8.3. Tissue–specific promoters and inducible promoters

Tissue-specific promoters (TSPs), a powerful tool for decreasing the toxicity of cancer gene therapy to normal tissues, have been used as targeted gene therapy approach. TSPs have been utilized for specific mutation compensation or delivery of prodrug-converting enzymes and also for controlling crucial viral replication regulators and consequent restriction of replication to tumor cells [118]. The safety and contingency of this approach has been shown in some initial clinical trials [119]. Of these, the cytomegalovirus (CMV) immediate-early promoter is often harnessed in gene therapy since it can express target genes at high levels in tumor cells. Lin et al. (2001) examined the effects of the involucrin (INV), keratin 14 (K14) and CMV promoters on the expression of the reporter gene beta-galactosidase. They introduce the plasmid DNA to BALB/c mice using a gene gun, and examined the skin biopsies. They found that the K14 and INV promoter constructs could induce the beta-galactosidase gene expression only in the epidermis, while the CMV promoter was able to elicit gene expression in both the dermis and epidermis [120]. To increase promoter strength while maintaining tissue specificity, Qiao et al. (2002) constructed a recombinant adenovirus encompassing a binary promoter system with a tumor-specific promoter carcinoembryonic antigen (CEA) driving a transcription transactivator with capability to express a HSV-TK. After successful application in vitro, they employed noninvasive nuclear imaging using a radioiodinated nucleoside (fraluridine (FIAU)) serving as a substrate for HSV-TK in BALB/C mice model. The results indicated the accumulated radioactivity only in the area of CEA-positive tumors after intratumoral injection, in which significantly less spread was observed to the adjacent liver tissue [121]. In another study, a vector with the human minimal tyrosinase promoter and two human enhancer elements (2hE-hTyrP) was compared with different hybrid promoter constructs containing tyrosinase regulatory sequences and the viral simian virus 40 (SV40) promoters. The hybrid SV40-based promoters were effective in vitro, and the in vivo tissue specificity of the 2hE-hTyrP vector was demonstrated in subcutaneous xenografted tumors model [122]. Another plausible approach to specifically target tumor cells for gene expression is to harness promoter elements that become activated in chemotherapy-resistant tumor cells [123]. In addition to TSPs, inducible promoters (IPs) can be exploited to minimize target gene expression in normal

cells. Harnessing the IPs, the timing of the gene expression can be modulated and controlled. Of a large number of inducible systems developed, only a few were translated into clinical gene therapy trials, including radiation-inducible genes [124]. Using this cancer gene therapy modality, promoters of radiation-inducible genes are exploited to drive transcription of transgenes in response to radiation, resulting in increased responsiveness of cancer cells to radiotherapy. These constructs, delivered by adenoviral vectors, can activate a transgene encoding a cytotoxic protein in tumor cells, in which the tumoricidal effects can be then localized temporally and spatially by X-rays. Perhaps, TNFerade (GenVec, Inc) is the best paradigm, which is an adenoviral vector containing radiation-inducible elements of the early growth response-1 promoter upstream of a cDNA encoding human TNF-α [125]. It has been translated into several clinical applications, e.g., as the first-line treatment of locally advanced pancreatic cancer in combination with 5-FU and radiotherapy [126]. However, it has not been approved.

9. Translational hurdles

Over the last couple of decades, various gene-based medicines have been developed in vitro with great potential to be translated for in vivo uses in clinic. It has been evinced that the gene therapy approach by virtue carries a certain degree of risk, thus the design and development of such modality need to meet the entire scientific and regulatory requirements. Some of the risks are procedural hazards (e.g. for parenteral medicaments), while some others happen to be specific to the genomedicine per se (e.g., immunologic reaction of viral vectors or nonspecific impacts of the delivered genes). All scientific, ethical, legal and social implications of this novel modality to genetic disease are involved for its successful translation. Fig. 7 schematically epitomizes the complexity of the steps for development and translation of gene-medicine for immunogene therapy of ovarian cancer.

In immunogene therapy, for example, stimulation of the cellular immune system to recognize and obliterate the cancer cells with genes encoding a variety of cytokines (e.g., interleukin-2 (IL-2), granulocyte - macrophage colony-stimulating factor (GM-CSF), co-stimulatory molecules such as CD80 and CD86) can 1) increase the immunogenicity of the transfected autologous tumour cells, 2) increase the likelihood of generating a tumor-specific cytotoxic T-lymphocyte (CTL) response. In the ex vivo transfection of antigen-presenting cells (APCs), the cells are transfected with a gene encoding a tumor-specific antigen (e.g., carcinoembryonic antigen, CEA) that is presented by major histocompatibility complex (MHC) class I molecules to antigen-specific CTLs via the T-cell receptor (TCR). Stimulated CTLs can find and eradicate the residual CEA-expressing tumor cells, in which GM-CSF can increase the activation of APCs and their migration into the tumor microenvironment [1].

Further, some degree of knowledge of industrial drug development is critical for innovation in this new sector, while healthcare systems and industries need to undertake more translatable approaches to fasten the in terms of cancer gene therapy.
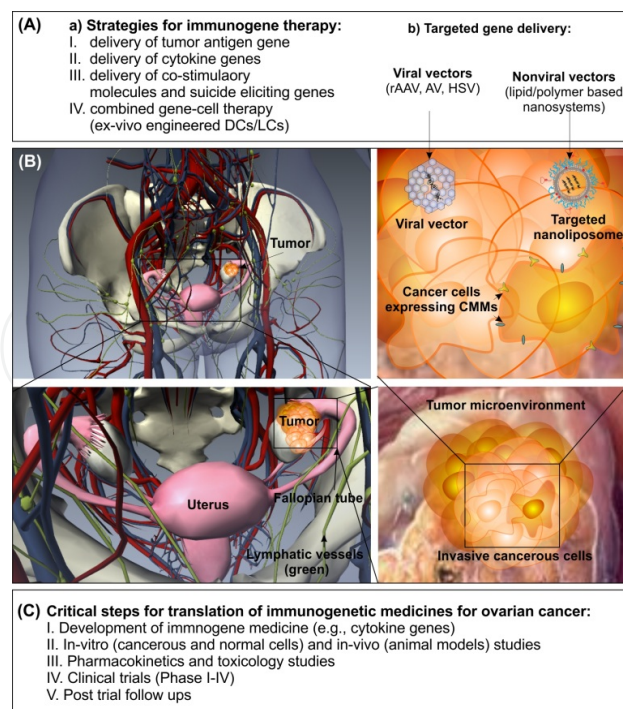
Figure 7. Schematic representation of translational approach for immunogene therapy of ovarian cancer. A) Design of the immunogene therapy based on a) different strategies of gene therapy and b) different gene delivery systems. B) Anatomical and cellular complexity of the ovarian cancer cells that need to be specifically targeted using advanced gene delivery systems and cancer marker molecules (CMMs). C) Various stages for translation of immunogenetic medicine.

10. Final remarks

Cancer gene therapy continues to grow even though clinical applications of this approach demand further investigations. Trajectory of gene therapy shows great impacts of genomedicines (i.e., As-ODNs, siRNA, Ribozymes, DNAzyme) both cell based and animal models, while tumor antigen-specific vaccines and DNA vaccines appear to be the most promising modalities. While suicide gene therapy, immunogene therapy and angiogenic gene therapy continue to become a mature modality, integration of nanotechnology toward development of multifunctional nanoparticles appear to provide a resilient, yet versatile, platform for targeted cancer gene therapy as "nano-genoceuticals". Rise of MSCs based cancer gene therapies may also open a new chapter as "cyto-genoceuticals". More than 65% of the gene therapy trials have been devoted to the cancer diseases; however, less than 3% of these trials have been progressed to the phase II/III stages and only few to the phase IV stage. The first approved cancer vaccination (Sipuleucel-T) has resulted in great clinical corollaries. Still many tumor suppressor and apoptosis-inducing genes can be evaluated for clinical applications. Attributable to intricate nature of malignant diseases, to achieve more effective gene therapy against cancer, genomedicines need to be advanced to be able to holistically target the most cancer causing genes. It is also essential to target both the tumor cells and other cancer associated players of the tumor microenvironment including: tumor microvasculature and tumor associated cells, stromal cells and CSCs.

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