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Vectors and strategies for nonviral cancer gene therapy

Jessica Pahle and Wolfgang Walther

Experimental and Clinical Research Center, Charité University Medicine Berlin and Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

SUMMARY

Introduction: This review presents recent developments in the use of nonviral vectors and transfer technologies in cancer gene therapy. Tremendous progress has been made in developing cancer gene therapy in ways that could be applicable to treatments. Numerous efforts are focused on methods of attacking known and novel targets more efficiently and specifically. In parallel to progress in nonviral vector design and delivery technologies, important achievements have been accomplished for suicide, gene replacement, gene suppression and immunostimulatory therapies. New nonviral cancer gene therapies have been developed based on emerging RNAi (si/shRNA-, miRNA) or ODN.

Areas covered: This review provides an overview of recent gene therapeutic strategies in which nonviral vectors have been used experimentally and in clinical trials. Furthermore, we present current developments in nonviral vector systems in association with important chemical and physical gene delivery technologies and their potential for the future.

Expert opinion: Nonviral gene therapy has maintained its position as an approach for treating cancer. This is reflected by the fact that more than 17% of all gene therapy trials employ nonviral approaches. Thus, nonviral vectors have emerged as a clinical alternative to viral vectors for the appropriate expression and delivery of therapeutic genes.

1. Introduction

The first gene therapies were developed for the treatment of genetic (primarily monogenic) diseases; these attempts were quickly exploited in experimental approaches to the treatment of cancer. Currently, more than 64% of all gene therapy trials worldwide are aimed at the treatment of solid or hematological malignancies.[1] This has made cancer gene therapy a dominant area in both basic and clinical research. A range of viral and nonviral vectors has been developed, and various strategies are being used as approaches to the treatment of malignant diseases. [2,3] In particular, nonviral systems have reached a new stage of development based on new types of vectors, including minicircle or sleeping-beauty vectors, improved transfer technologies such as nanoparticles/lipofection, and physical technologies (e.g. sonoporation, electroporation, particle bombardment/gene-gun, jet-injection). In terms of interfering with cancer biology, a range of therapeutic strategies have shown significant progress over the past few years; these include approaches based on immunogenes, suppressor genes, or gene replacements, gene-directed enzyme–prodrug/suicide gene therapies, gene suppression, or oncolytic virotherapies. The ultimate success of an approach requires not only the availability of an appropriate therapeutic transgene, but also vectors that achieve efficient gene transfer and expression.

The limitations of viral vectors (e.g. insertional mutagenesis, immunogenicity, etc.) have given rise to a renaissance in the development of nonviral alternatives.[3] This is mainly due to recent developments that allow nonviral vectors to efficiently express genes while exhibiting low toxicity and immunogenicity at significantly improved levels of transfer. Furthermore, they are safer and cheaper to produce, making them attractive for development into potential clinical applications. The biggest challenge, as for viral vectors, remains their applicability in vivo. The decisive factors for successful gene transfer are vector stability, protection from degradation, and proper targeting. Two major strategies have been pursued: the development of chemical or physical delivery systems, and methods of local delivery for nonviral vectors.

Poor selectivity and transfer efficiency, especially in clinical application, represent the limiting factors for
Successful gene therapy and are therefore in focus for experimental and clinical efforts. This is even more important, as cancer is a metastasizing disease, which could much better treated if metastatic lesions can be efficiently targeted. Here proper tumor-targeting of nonviral vectors is one decisive requirement. In this context, nonviral gene therapies, which aim for the antitumoral immune stimulation via DNA- or RNA-vaccination strategies, are promising approaches to combat cancer metastases. Alternative strategies aim at local gene therapy of cancer for local treatment of the disease. In fact, about 20% of all clinical cancer gene therapy trials (Table 1) are performed as local viral or nonviral gene transfers. [4] This concept is still attractive, however, since direct antitumoral effects can be achieved, which can be further improved by potential bystander or immunostimulatory effects.

2. Strategies used for cancer gene therapy (Figure 1)

Most strategies that are currently used for the treatment of cancers do also employ nonviral vectors for gene delivery and proper transgene expression. Due the tremendous improvements achieved in nonviral vector design, they are employed in all important gene therapeutic strategies to treat cancer diseases.

2.1. Gene correction therapy

The initial goal of gene therapy was to provide gene correction of defective genes. This was adapted to the treatment of malignant diseases in order to replace mutated genes of oncogenic potential and to therefore inhibit the malignant phenotype of cancer cells. It turned out that this strategy had only a limited impact on cancer gene therapy, but has potential if combined with chemo- or radiotherapy. Recent strategies aim to restore tumor suppressor gene expression: this strategy harbors potential for cancer therapy. For example, such promising strategies utilize repeated systemic delivery of liposomally encapsulated plasmid DNA (pDNA). In one study, the tumor suppressor gene TUSC2 was administered to cancer patients by intravenous injection. The authors demonstrated transgene expression in primary and metastatic lung tumors associated with alteration of TUSC2-regulated apoptotic pathways.[8] In this study, five patients with stable disease and two with minor response were reported. Alternatively, another study employed anti-transferrin-targeted systemic liposomal nanodelivery of p53-expressing plasmid.[9] The study demonstrated tumor-selective p53 expression in primary and metastatic biopsies and reported stable disease and intratumoral necrosis for some patients.

2.2. Suicide gene therapies

Suicide gene therapy has been developed particularly for the treatment of cancer and has long translated to Phase I and II clinical trials. Numerous prodrug-convert- ing enzymes of bacterial or viral origin were cloned and used for expression in tumor cells. These enzymes are able to convert nontoxic prodrugs into toxic metabolites, subsequently killing tumor cells and also neighboring cells via the bystander effect. The most prominent members of these suicide genes are the cytosine deaminase (CD), Herpes simplex virus thymidine kinase (HSV-tk), cytochrome P450-2B1 and nitroreductase, as well as genetically modified variants of these suicide genes.

Recent developments with respect to the aforementioned suicide genes aimed at optimization via mutated variants or the functionality of fusion proteins. These developments allowed for either a more efficient conversion of the toxic metabolites or for the modification of substrate specificity to use alternative prodrugs for cancer treatment.[10] Other approaches used suicidal capacities of bacterial or plant (e.g. saporins) toxins for gene therapy. A recent example is the pore-forming Clostridium perfringens enterotoxin (CPE), which selectively killed claudin-3 and -4 overexpressing epithelial tumor cells in vitro and in vivo.[11] Such studies pointed to the emerging potential of toxin gene therapy for tumor eradication.
<table>
<thead>
<tr>
<th>Vector Type</th>
<th>Delivery system</th>
<th>Chemical</th>
<th>Physical</th>
<th>Delivered gene</th>
<th>Cancer entity</th>
<th>Phase of clinical trial</th>
<th>Reference</th>
</tr>
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<td>Vector plasmid</td>
<td>Naked DNA</td>
<td>Intraperitoneal injection</td>
<td>DTA-H19</td>
<td>Ovarian cancer</td>
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<td></td>
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<td>AMEP</td>
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<td>Bladder cancer</td>
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<td>Multiple myeloma</td>
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<td>Plasma cell leukemia</td>
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<td>Intradermal jet-injection</td>
<td>EGFR</td>
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<td>TGF-β2</td>
<td>Advanced cancers</td>
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<td>c-raf-1</td>
<td>Advanced solid tumors or lymphoma</td>
<td>Phase II</td>
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<td>c-raf-1</td>
<td>Liver metastases</td>
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<td>c-raf-1</td>
<td>Breast cancer</td>
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<td></td>
<td></td>
<td></td>
<td>c-raf-1</td>
<td>Advanced non-small lung cancer</td>
<td>Phase II</td>
<td>[7]</td>
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</table>

(Continued)
In addition to the known suicide gene technologies, new suicide-concepts have entered the field, such as the suicide gene therapy introduced by DiStasi et al. This strategy was based on use of modified caspase-9 for apoptosis induction.\[12\] They expressed the modified caspase-9 (iCasp9) as an inactive subunit linked to the FK506-binding protein FKBP12, which only mediated apoptosis after dimerization, through the small molecule AP1903. This dimerization induced rapid cell death following AP1903 application. This novel suicide system has already been used in a clinical study in which five patients received engineered T-cells for adoptive cell therapy. As graft-versus-host disease (GVHD) symptoms were seen in one patient, application of AP1903 eliminated these T-cells by 90%. Therefore, such a system is of most value for T-cell or other

Table 1. (Continued).

<table>
<thead>
<tr>
<th>Vector Type</th>
<th>Delivery system</th>
<th>Targeted molecule</th>
<th>Cancer entity</th>
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<td>STAT3</td>
<td>Head and neck cancer</td>
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<td>Aptamer</td>
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<td>Intravenous injection</td>
<td>Nucleolin</td>
<td>Advanced solid tumors</td>
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<td>siRNA</td>
<td>Lipid nanoparticle ALN-VSP02</td>
<td>Intravenously injection</td>
<td>VEGF and KSP protein kinase N3</td>
<td>Advanced solid tumors with liver involvement</td>
<td>Phase I</td>
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<td></td>
<td>Liposomal particle Atu027 PEG Nanocomplex CALAA-01</td>
<td></td>
<td>M2 subunit of ribonucleotide reductase</td>
<td>Advanced solid cancer</td>
<td>Phase I</td>
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<tr>
<td></td>
<td>Polymer siG12D LODER</td>
<td></td>
<td>KRAS</td>
<td>Solid tumor cancers</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

DTA: Diphtheria toxin A; IL-12: Interleukin-12; AMEP: Antiangiogenic metargidin peptide; pVAXrCPSAv53I: rhesus prostate specific antigen (PSA); tetwtCEA: human carcinoembryonic antigen (CEA) fused to a tetanus toxoid T helper epitope; fus1: tumor suppressor gene fus1; DOTAP: N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate; PEI: Polyethylene glycol; CYL-02: Mouse somatostatin receptor subtype 2 and fusion protein of human deoxyctydine kinase and uridine monophosphate kinase; MIDGE: Minimalistic Immunogenically Defined Gene Expression; hTNF-alpha: human tumor necrosis factor alpha; dSLIM: double Stem Loop ImmunoModulator; MGN: Mologen; ASO: Antisense Oligonucleotides; EGFR: Epidermal Growth Factor Receptor; 2'-O-MOE-PS; 2'-O-methoxymethyl; Hsp27: Heat shock protein 27; STAT3: Signal transducer and activator of transcription 3; DLBCL: Diffuse Large B-Cell Lymphoma; TGF-β2: Transforming Growth Factor beta 2; HIF-1α: Hypoxia-Inducible Factor 1-alpha; Bcl-2: B-cell lymphoma 2; c-raf: Raf proto-oncogene serine/threonine-protein kinase; Raf: rapidly accelerated fibrosarcoma; VEGF: Vascular Endothelial Growth Factor; KSP: Kinesin Spindle Protein; KRAS: V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog.

Figure 1. Scheme of the major strategies in cancer gene therapy. The different strategies in gene therapy either aim at stimulation of the immune response against the tumor or at the tumor killing as well as intervention of target gene expression to reduce e.g. tumor growth or tumor resistance.
cell-based therapies to better control cell function via such a molecular safety-switch.

2.3. Gene suppression and gene silencing therapies

Gene suppression therapies, which aim at intervention of gene transcription and translation, are of growing importance. Both antisense oligodeoxynucleotides (AS-ODNs) and small interfering (si) RNA represent the emerging class of targeted nucleic acid-based pharmaceuticals, which are in early clinical development. Transcripts of genes with different cellular function have been targeted by AS-ODN strategies such as c-raf (raf proto-oncogene serine/threonine-protein kinase), c-fos (proto-oncogene, human homolog of retroviral oncogene v-fos), c-myc (proto-oncogene c-myc), H-ras (Harvey rat sarcoma viral oncogene homolog), Her2/neu (human epidermal growth factor receptor 2), bcl-2 (B-cell lymphoma 2), VEGF (vascular endothelial growth factor), Ang-1 (angiopoietin-1). It was demonstrated that AS-ODNs successfully inhibited gene expression, leading to tumor growth inhibition or chemo- as well as radio-sensitization.[13–15] Recent Phase I/II trials aimed at suppressing bcl-2 (G3139) in addition to a Phase I study suppressing c-raf-1 (ISIS 5132), which demonstrated improved patient sensitivity toward chemotherapy (Table 1).[6,7]

Another emerging gene suppression strategy utilized siRNA technology as an approach for sequence-specific target gene suppression. As for other gene therapies, siRNA stability and proper delivery are decisive factors for successful treatment. Recent developments have suggested that nanocomplexes (organic polymeric or inorganic nanoparticles) and physical transfer technologies may overcome stability and delivery issues.[16] One approach used ultrasound-targeted microbubbles for efficient anti-EGFR siRNA in vitro and in vivo, which demonstrated reduced tumor growth in a squamous cell carcinoma model.[17] Another recent example demonstrated that intravenous administration of liposomal siRNA formulation (Atu027) targeted at the protein kinase N3 (PKN3) expression efficiently inhibited tumor progression and metastasis formation in lung and mammary carcinoma models.[18] This approach was meanwhile brought to the clinic for the treatment of patients with advanced solid tumors (Table 1).[19] The rapidly growing number of experimental studies and clinical trials, which are based on siRNA technologies, underline the potential for specific cancer gene therapies in local as well as in systemic applications.

2.4. Oligodeoxynucleotide decoy therapy for transcription factor targeting

The use of oligodeoxynucleotides (ODNs) for transcription factor decoy represents a novel therapeutic strategy for gene suppression therapies. This strategy is based on the competitive inhibition of transcription factors by double-stranded ODNs, which are composed of transcription factor recognition sequences. ODNs compete for transcription factor binding, leading to transcriptional inhibition of target gene expression regulated by a particular transcription factor. In vivo studies have shown that ODN decoy strategy generated antitumoral effects via transcriptional targeting of STAT3, a well-known oncogenic transcription factor in a lung cancer xenograft model.[20] This strategy has already been used for treatment of patients suffering from head and neck cancer.[21] The intratumoral injection of the STAT3 decoy ODN led to a reduction in target gene expression (Bcl-XL, cyclin D1). Additional in vivo studies with systemic application of improved, more stable STAT3 decoy ODNs showed reduced growth of head and neck tumor xenografts in mice, paralleled by reduced target gene expression. This novel approach demonstrated the great potential of such decoy therapies to target oncogenic transcription factors. Furthermore, combination approaches using decoy therapies together with chemo- or radiotherapy might improve the therapeutic efficacy.

2.5. miRNA-targeted cancer therapies

miRNAs represent attractive targets for gene therapeutic approaches to treat cancer.[22] Since their discovery almost two decades ago, numerous miRNAs have been identified with important impact on tumor development, progression, metastasis formation (oncogenic miRs: e.g. miR-21, miR-29), tumor suppression (e.g. let-7, miR-34), or therapeutic response (e.g. miR-1, miR-21, miR-181a). miRNA cancer therapy is currently experiencing accelerated growth. One study employed miR-181a transfer in vitro and in vivo to target the ABCG2 resistance gene.[23] The study demonstrated that even in vivo Mitoxantrone-resistant MCF-7 xenotransplant tumors are resensitized by miR-181a therapy. In another approach, anti-miR-21 siRNA was loaded onto a graphene carrier to successfully downregulate ABCB1 expression and sensitize adriamycin-resistant MCF-7 cells toward this drug.[24]
2.6. DNA and RNA vaccination therapy

Due to the fact that cancer represents a systemic disease with potential metastasis formation, vaccination strategies are attractive to combat them via systemic activation of the anticancer immune response. The recombinant vaccines (usually naked DNA vectors expressing tumor-associated antigens; TAAs) for local (intradermal, intranodal) or systemic application are used for stimulation of the antitumor immune response.[25] In this regard, gene therapy is employed to express TAAs (e.g. Her2/neu, MART1 (melanoma antigen recognized by T cells 1), PSA (prostate-specific antigen), CEA) and has been translated into clinical trials.[26] Among a variety of antigens, p53 is attractive since it is inactivated/mutated in many tumors. Contrarily, the p53 protein is of low immunogenicity, which creates hurdles in use of p53 as an antitumor vaccine. To solve this dilemma, Soong et al. utilized human p53 to xenogenically vaccinate C57BL/6 mice, and were able to induce anti-p53 immune response with the involvement of p53-specific antibodies and CD8+ T-cell precursors.[27] More importantly, these animals were protected against tumor challenges with p53-expressing MC38 murine colon carcinoma cells. This DNA vaccination study is therefore an important step toward successful antitumoral vaccination protocols to treat metastatic diseases, based on use of xenogenic DNA vaccines. In the last decade, the establishment of DNA-based vaccines was paralleled to the development of RNA vaccines, mainly as mRNA vaccines.[28] Here, mRNA-encoding particular tumor antigens (e.g. WT1, CEA, hTERT, PSA etc.) or autologous tumor-derived mRNA is used for genetic modification of dendritic cells (DCs) by intradermal or intranodal application.[29,30] These engineered DCs are capable of inducing antitumoral immune responses via activation of CD8+ and CD4+ cells. In fact, this has also been achieved by the direct mRNA-vaccination of renal cell carcinoma for stimulation of antitumoral T-cell responses against different antigens (MUC1, Her-2/neu, Mage-A1, gp100, etc.).[31] Such RNA vaccinations have already been tested in clinical trials for the treatment of melanoma and prostate-, lung-, breast, and colon cancer patients.[32] This development of clinical applications is supported by the achievements made for stabilization and expression optimization of the mRNA vaccines.[28]

3. Nonviral vector systems (Tables 1 and 2)

3.1. Plasmid vectors

Plasmid vectors represent an important platform for gene delivery, as they are safe, stable in storage, easy to manipulate, and comparatively inexpensive to produce. However, the gene transfer efficiency of plasmid vectors is low. Thus, there is great focus on the improvement of delivery and optimization of pDNA for enhanced cellular uptake and increased gene expression. Plasmid vectors are small, circular double-stranded DNA molecules capable of replicating in bacterial host cells. Their size ranges from 0.8 to 120 kbp and the capacity for transgene DNA is almost unrestricted.[53] Recent developments aim to significantly reduce bacterial backbone sequences to the essential minimum and to generate pDNA of defined topologies (open circular vs. closed circular and supercoiled) for improved cellular uptake. Unfortunately, naked pDNA vectors are vulnerable for degradation and permit only transient, episomal gene expression. Therefore, modifications have been carried out to improve stability, persistence, and the targeting ability of pDNA. For example, the replacement of polyA with synthetic or SV40 polyA sequences was shown to extend the half-life of supercoiled pDNA.[2] Furthermore, the selection of the promoter can be crucial for transgene expression, as tissue-specific promoters may permit improved transcriptional efficacy and selectivity.[54]

| Table 2. Overview of the approximate sizes of major classes of nonviral vectors. |
|-----------------------------------------------|---------|-------|-------------------|-------------------|
| Nonviral vector systems                      | Subspecies | Size (bp) | Delivery shape               | References |
| Plasmid DNA (pDNA)                           | ~1–250 kbp  | Double-stranded  | [8,9,11,33] |
| Miniplasmid/mini-intronic plasmid            | ~5 kbp     | Supercoiled, circular DNA | [34,35] |
| Minicircle                                   | ~4 kbp     | Supercoiled, circular DNA | [36–38] |
| Dumbbell-shaped Minigene, SLIM               | ~0.1–25 kbp | Linear DNA covalently closed | [5,39] |
| Oligonucleotides                             | ~10–20 bp  | Chemically modified 3’-5’ deoxy – or ribonucleotides | [13–15,40–42] |
| RNAi                                         | ~19–29 bp  | Double-stranded DNA or RNA sequence | [46–48] |
| shRNA                                        | ~20–24 bp  | Double-stranded RNA | [49] |
| siRNA                                        | ~15–22 bp  | Double-stranded stem loop RNA | [22–24,51,52] |
Due to the lack of integration and silencing processes, the expression of transgenes from pDNA is transient. To overcome this, transposon/transposase-based systems were designed to offer high transgene capacity and expression stability. Transposons are DNA sequences that are able to move from one genomic location to another and are used to introduce foreign DNA into a host genome, thereby increasing the longevity of gene expression.[55] They consist of the transposase gene flanked by two terminal inverted repeats (TIRs). Recognition of those TIRs by transposase leads to excision of the transposon DNA body, which is then inserted into a new genomic location. To date, different systems, such as sleeping beauty (SB), frog prince, mariner, minos, Tol2, and piggyBac (PB), are known to be active in a mammalian cell. Of those, the SB system has been well characterized and is most widely used as a genetic tool.[56] The SB system consists of two major components: a transposon, composed of inverted repeats (IRs) that carry the gene of interest, and a transposase enzyme. It can be used to create both, gain-of-function and loss-of-function mutations and has wide implications for somatic gene therapy insertional mutagenesis and functional genomics. Using SB systems, oncogenes and tumor suppressor genes involved in tumorigenesis have been identified in different cancer entities, such as glioblastoma,[57] gastrointestinal cancer,[58] osteosarcoma,[59] and B-cell lymphoma.[60] Beyond that, the SB system can be adapted for human cancer gene therapy, as has been shown in different in vitro and in vivo studies.[61] For example, CD19-CAR-specific T-cells[62–64] and IL-11Ra-CAR-specific T-cells[65] engineered using the SB transposon system revealed promising application in treating lymphoid malignancy or osteosarcoma pulmonary metastases. Furthermore, SB systems can also be used to directly transfer therapeutic genes in vivo for cancer treatment. This was shown for a modified SB transposon containing an angioatin-endostatin fusion gene (StatinAE), which was applied in a mouse model of colorectal cancer (CRC) and which led to significant growth inhibition of liver metastases. This study emphasizes the potential of using nonviral SB transposon systems for effective treatment of CRC.[33] In 2013, the first clinical trial of SB-mediated gene therapy was initiated to treat leukemia or lymphoma patients, with CAR-engineered T-cells implicating the potential of transposon-based immunogene therapy.[66]

During the past decade, several other systems have been developed and optimized. A noteworthy example is the PB transposon/transposase system, which is capable of achieving long-term, stable transgene expression and delivering of large transgenes or multiple genes.[67] Current studies support the nonviral use of a transposon system for generating genetically modified T-cells for clinical use to treat B-lineage malignancies or Her2-positive brain tumor cell line.[34,68]

Another approach employs scaffold/matrix attachment regions (S/MARs) to prolong transcription, promote episomal maintenance, and overcome epigenetic silencing of pDNA. S/MARs are short AT-rich genomic DNA sequences that interact with the nuclear matrix by generating chromosome domains, arranging the DNA within the nuclear matrix, and modulating DNA replication during cell division, resulting in prolonged gene expression independent from the integration site.[69,70] It has further been published that vectors harboring S/MAR DNA elements linked to an expression cassette are able to recruit nonviral factors to promote episomal replication and mitotic stability.[34,71] To date, episomal plasmids containing S/MARs have been successfully transfected into cervical,[72] hepatocellular-, and pancreatic carcinoma cells[73] as well as leukemia and glioma cells,[74] where they revealed tissue-specific replication and maintenance as extra-chromosomal episomes with less side effects. The promising potential of S/MAR-based vector system has further been shown in the treatment of chronically HBV-infected patients, as the HBV replication was long-term suppressed by the expression of suited short hairpin (sh) RNAs.[75]

Plasmid-based vectors are also used to deliver shRNA, an artificial RNA duplex with a tight hairpin turn, to provide specific, long-lasting gene silencing, as they are constantly synthesized in the host cells when efficiently transfected. This DNA vector-mediated RNAi is very attractive if combined with cell-type specificity by selective promoters. The shRNA technology offers a great opportunity for the nonviral treatment of cancer, as it was demonstrated for the delivery of c-Myc shRNA, which led to target gene silencing, inducing mammary tumor growth inhibition and increased animal survival in Brca2/p53-mutant mice.[49]

Due to the aforementioned pDNA modifications and optimization, nonviral gene therapy approaches have been considerably improved. However, the use of antibiotic resistance genes as selection markers for plasmid production still raises safety concerns for the clinical use as they could provoke allergic reactions. Therefore, new antibiotic-free selection systems, such as plasmid free-of-antibiotic resistance (pFAR) or RNA OUT, have been designed to increase the biosafety of nonviral gene therapy trials. In a recent study, an increased transgene expression level was obtained after electrotransfer of a pFAR plasmid into muscle, skin, or tumor, suggesting
that use of improved gene vectors could lead to a more effective cancer therapy.[76]

3.2. Minicircle

Conventional plasmids contain numerous genetic elements, which are necessary for pDNA production, and are dispensable for gene therapeutic use. These include pro-ad eukaryotic selection markers, promoters and enhancers outside the desired expression cassette, and the origin of replication as well as unmethylated CpG islands (5’-CG-3’ dinucleotides) of the backbone. Each of them affects the biological safety, such as uncontrolled immune stimulation or possible alteration of the gene expression profile. It has further been shown that the bacterial backbone DNA can also lead to expression silencing of transgenes. Accordingly, efficiency and safety can be improved by backbone removal from pDNA, by in vivo site-specific recombination.[78] To achieve this, expression cassette of the parental plasmid is flanked by two recognition sites of site-specific recombinase. The in vivo expression of recombinase divides the parental plasmid into two supercoiled molecules, a replicative miniplasmid and a minicircle. This supercoiled, circular minicircle DNA is free of prokaryotic sequence elements and significantly reduced in size, ~4 kb, thereby enhancing gene expression efficiency and allowing for a more robust and persistent gene expression.[36] Moreover, lack of bacterial sequences increases their safety for therapeutic use due to the reduced number of pro-inflammatory unmethylated CpG motifs.[37] Previous studies demonstrated the promising use of minicircles for a safe and efficient nonviral gene therapy. For example, to treat nasopharyngeal carcinoma, minicircle-mediated interferon (IFN)-gamma gene therapy was used, leading to an antiproliferative effect on cancer cells in vitro and a profound antitumoral effect in vivo.[38]

Very recently, another transgene expression vector, the mini-intronic plasmid (MIP), came into focus. MIPs contain a bacterial replication origin and a selectable marker that maintain the juxtaposition of the 5’- and the 3’-ends of the transgene expression cassette, comparable to a minicircle. Both minicircle and MIP can overcome transgene silencing occurring with plasmid vectors and the MIP system additionally provides higher expression levels in vivo and in vitro. These improved plasmids in combination with the aforementioned antibiotic-free selection system could be beneficial to all gene transfer and gene therapy approaches.[34,35]

3.3. Dumbbell-shaped minimalistic vectors

Dumbbell-shaped minimalistic vectors, such as the minimalistic immunologically defined gene expression (MIDGE) vectors, currently represent the smallest available vector system of protein encoding expression cassettes and are characterized as double-stranded linear covalently closed (dumbbell) DNA molecules. In contrast to minicircle or pDNA vectors, they consist only of the respective expression cassette, including promoter, the transgene, and polyA signal.[79] This structure highly improves the transcriptional efficiency, safety, and has demonstrated promising application in vitro and in vivo. The production of these vectors is comparatively simple as the expression cassette can be amplified by PCR or can be excised via specific restriction sites from plasmid, followed by subsequent ligation of short hairpin ODNs for end-sealing. The covalently closed loops protect the vector from degradation by nucleases and allow additional modifications for adaption to specific applications.[80] In this regard, peptides or carbohydrates can be linked to the vector as ligands to target specific cells or tissues. Minimalistic vectors have successfully been used in various tumor cell types, such as colon carcinoma, melanoma, or leukemia in vitro and in vivo.[5] Moreover, MIDGE vectors have entered clinical use for cancer gene therapy and immunostimulatory (SLIM vectors) approaches (Table 1).[39] Due to their unique structure and especially their reduced size, such minimalistic vectors are of high clinical potential for nonviral gene therapies.

3.4. Oligonucleotides

In the last decades, oligonucleotides have attracted attention as they provide an effective way to design sequence-specific ligands for nucleic acids or DNA-binding regulatory proteins for selective interference of gene expression. Oligonucleotides are used to deactivate or silence genes that are involved in pathogenesis.

3.4.1. Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short, single-stranded nucleotide analogs, complementary to and can selectively target mRNA. Hybridization of ASOs to respective mRNA leads to specific inhibition of gene expression by various mechanisms. The ASO-mRNA heteroduplex either triggers RNase H activity, leading to degradation of mRNA, induces translational arrest of ribosomal activity, interferes with mRNA maturation, or destabilizes pre-mRNA in the nucleus, which results
in reduced translational activity of the target transcript. [81] Various chemical modifications, such as phosphorothioate, 2′-O-methyl (2′-OMe), 2′-O-methoxethyl (2′-MEO) and gapmers have been developed to improve nuclease resistance, increase efficiency and affinity, prolong half-life within the tissue, and reduce toxicity. For further enhancements, modifications of the furanose ring were done and peptide nucleic acid (PNA), locked nucleic acid (LNA), and phosphorothioamide morpholino oligomer (PMO) came into focus, leading to greatly enhanced cellular uptake and hybridization affinity, high biostability, and excellent nuclease resistance. [51] Considering these aforementioned features, ASOs are highly valuable therapeutics to treat cancer, as they are capable of successfully inhibiting gene expression, leading to tumor growth inhibition.[13–15,40,41] In one recent study, it was demonstrated that ASO blocking the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) prevented metastasis formation after tumor implantation.[42] Thus, targeting specific markers with ASO could provide a potential therapeutic approach to prevent metastasis.

### 3.4.2. Aptamers

Aptamers are short, artificial, single-stranded DNA or RNA sequences folded into secondary and tertiary structures, which are able to recognize and bind to a wide range of targets, including amino acids, drugs, proteins, or cells. They are also considered a novel class of compounds with diagnostic and therapeutic potential and may even substitute antibodies. Synthesized through an in vitro selection and amplification process, known as SELEX (systematic evolution of ligands by exponential enrichment), a wide range of DNA and RNA aptamers have been generated during the last decade.[82] Due to their small size and strong target affinity, as well as their lack of immunogenicity and easy modification, these oligonucleotides represent a valuable nonviral therapeutic and targeting class of entities. The target guiding potential of aptamers has been demonstrated in the in vitro and in vivo approaches for the use of the DNA aptamer AS1411. By systemic application through targeting the protein nucleolin expressed on MV4-11 leukemia cells, the AS1411 aptamer permitted effective binding to nucleolin and cellular uptake of the aptamer and antitumoral effects via nucleolin binding.[46] Meanwhile, this approach was also successfully applied for the treatment of melanoma and renal cell carcinoma using AS1411 aptamer for tumor targeting and therapy.[47,48] In fact, aptamers also have great potential to efficiently treat metastasis via systemic application and selective targeting.

### 3.4.3. Decoy oligonucleotides

Another nonviral strategy uses artificial double-stranded decoy ODNs, which compete with endogenous cis-elements of the targeted gene. Once delivered to the targeted cells, decoy ODNs bind to a specific transcription factor, which leads to the transcription factor’s inability to bind to promoter region, subsequently leading to the removal of bound trans-factor from the endogenous cis-element. This results in significant reduction or blockade of transcriptional activity. [51] The antitumoral potential of these decoy ODNs has been demonstrated in numerous studies.[21,43–45] In one very recent in vitro approach, cationic solid lipid nanoparticles (SLNs) were used to encapsulate STAT3 decoy ODN. These complexes were efficiently taken up by human ovarian cancer cells, which led to significant inhibition of cell growth and cell invasion.[83] Another parallel in vivo study was performed to evaluate the effects of the liposome nuclear factor of kappa B (NF-κB) decoy ODNs on murine myeloma models. Treatment with liposome-NF-κB decoy ODN complex led to efficient suppression of NF-κB DNA-binding activity and inhibition of IL-6 expression. Further, the treated animals showed a better survival time and revealed smaller tumors, leading to the conclusion that the transfection of NF-κB decoy ODN may provide a new therapeutic strategy for multiple myeloma.[45] More importantly, the decoy ODN strategy has been used in the first clinical trial to treat patients suffering from head and neck cancer, demonstrating the applicability of this novel approach.[21]

### 3.4.4. siRNAs and miRNA as therapeutics

Another novel and potential development in oligonucleotide technology is the gene silencing potential of the siRNA. They are short, ~20–24 bp double-stranded RNA oligonucleotides mediating the degradation of complementary mRNA target after correct antisense strand has bound into RNA-induced silencing complex (RISC).[84] But as described before, unmodified siRNA also degrades within short time. Therefore, chemically modified analogs, such as 2′-OMe incorporation or backbone modification, with a better stability and efficiency, have been designed. The use of siRNAs for specific silencing of genes that are involved in disease pathogenesis holds promise for the development of a new class of therapeutics. Very recently, the first-in-human trial of siRNA targeting VEGF and kinesin-spindle protein (KSP) was performed in cancer patients with liver metastasis (Table 1).[50]
miRNAs represent another group of small noncoding RNAs, which are transcribed from several different loci in the genome. These genes encode for long RNAs with a hairpin structure, which when processed by RNase III enzymes form duplexes of 15–22 nucleotides with two-nucleotide overhang at the 3’ end. The mature miRNAs consist of guide and passenger strands with mismatches. These small, endogenous molecules can influence translation by binding to the complementary site on the target mRNA, where they either block or induce degradation of mRNA translation. miRNAs can be characterized as tumor-suppressing miRs, which are responsible for the inhibition of oncogenes, or as onco-miRs, which inhibit translation of tumor suppressor genes. If tumorigenic miRNAs are overexpressed, they can be further blocked with synthetic oligonucleotides known as anti-miRs, e.g. anti-miR221 for the treatment of breast cancer.

3.4.5. Other vector systems
The briefly described different nonviral vector systems are complemented by other vectors, which might provide some value for use in cancer gene therapy. In this context, human artificial chromosomes (HACs) could be of interest for gene correction approaches to interfere with the malignant phenotype of cancer cells. The HACs act as artificial chromosomes and provide the potential for regulated and long-term expression of transgenes.

Another interesting method of vector development combines the advantageous features of lentivirus and nonviral vectors in so-called hybrid vectors. These vectors are based on the use of engineered integrase-deficient lentivirus (IDLV) to reduce the risks associated with lentiviral integration. The IDLV can efficiently perform gene transfer followed by the generation of nonintegrated episomal linear molecules, which can then be circularized by target cell enzymes. These episomal molecules serve as efficient expression vectors for transient transgene expression. Such hybrid vectors are of potential interest for more efficient gene transfer to treat cancer.

4. Nonviral delivery systems
One key requirement for efficient nonviral gene therapy is the effective transfer of the vectors. Different nonviral delivery systems have been developed, including chemical and physical systems comprising either physical (needle injection, gene gun, electroporation, sonoporation, and magnetofection) or chemical methods (cationic lipids, polymers, and the combination of both), which are applied locally or systemically.

4.1. Chemical delivery systems and nanoparticles
Chemical nonviral delivery systems, generally classified into peptide-, polymer, or lipid-based and inorganic particles, represent powerful methods for successful gene transfer, as their efficacy has been shown in many in vitro studies as well as in vivo and clinical approaches (Tables 2 and 3). These vector systems

![Figure 2](image-url)
Table 3. Overview of chemical delivery methods.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Characterization/features</th>
<th>Pros</th>
<th>Cons</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic lipids (liposome)</td>
<td>Nucleic acids incorporated in lipids Transfection reagents: lipofectamine, fuge</td>
<td>Safe, low cytotoxicity</td>
<td>Low–medium efficiency, immunogenic, expensive</td>
<td>[8,9,88–90]</td>
</tr>
<tr>
<td>Combination of lipoplexes and polyplexes</td>
<td>Vector core of polycation DNA enveloped by lipid</td>
<td>High efficiency in vitro, easy to handle</td>
<td>Low–medium efficiency, immunogenic, expensive</td>
<td>[94,95]</td>
</tr>
<tr>
<td>Carrier materials: nanoparticle, biological materials, nanoeomulsions, microparticles</td>
<td>Nucleic acids or nonviral vectors are linked to these materials covalently or non-covalently</td>
<td>Physical and chemical properties of the carrier used</td>
<td>Low efficiency</td>
<td>[16,17,96–99]</td>
</tr>
</tbody>
</table>

Table 4. Overview of different physical delivery methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Pros</th>
<th>Cons</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Needle injection of naked DNA/RNA (pDNA, ODNs, etc.)</td>
<td>Safe, easy</td>
<td>Low transfection efficiency</td>
<td>[25]</td>
</tr>
<tr>
<td>Gene gun</td>
<td>Low cytotoxicity</td>
<td>Low efficiency, hallow penetration</td>
<td>[39,100]</td>
</tr>
<tr>
<td>Jet-injection</td>
<td>Needle-free, easy to control, safe</td>
<td>Tissue damage, moderate efficiency</td>
<td>[5,11,101,102]</td>
</tr>
<tr>
<td>Electroporation</td>
<td>Highly effective, good efficiency, transfer of large-size DNA</td>
<td>Accessibility for electrodes, surgical procedure required, tissue damage</td>
<td>[17,103–105]</td>
</tr>
<tr>
<td>Sonoporation</td>
<td>Noninvasive, efficient, safe</td>
<td>Low efficiency</td>
<td>[106–108]</td>
</tr>
<tr>
<td>Magnetofection</td>
<td>Noninvasive, precise targeting, low cytotoxicity</td>
<td>Transient transfection</td>
<td>[109–112]</td>
</tr>
</tbody>
</table>

address many challenges of gene transfer, such as extracellular stability, specific cell targeting, internalization, endosomal escape, nuclear envelope entry, nucleic acid release, and genomic integration.[113]

To date, some of the aforementioned challenges have been accomplished, as multiple vectors are achieving long half-lives due to stable carrier molecules and modifications to the surface of the lipoplexes with hydrophilic polymers, such as polyelectrolyte glycol (PEG) or the integration of pre-encapsulated drug-/gene-loaded liposomes within depot polymer-based systems.[114] Improved specificity and endosomal escape through the proton sponge effect have also been achieved. However, to reach clinical efficiency, nuclease-mediated degradation must be significantly reduced, accumulation at the site of interest maximized, and effective cellular internalization as well as intracellular trafficking to the nucleus has to be assured.

4.1.1. Cationic lipids

Cationic lipid-based vectors (liposomes) are among the most commonly used nonviral gene carriers, and hundreds of lipids have been developed for gene transfer, including the synthetic cationic lipids such as DOTMA (N-[1-(2,3-Dioleyloxy)propyl]-N,N,N-trimethylammoniumchlorid). All cationic lipids are characterized by three structural groups: a positively charged head group, a hydrophobic tail, and a linking group. Numerous modifications, such as transfer efficiency, stability, and low toxicity, can be conducted and DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate), DOTAP (1,2-Dioleyl-3-trimethylammonium-propan), DMRIE (1,2-dimyristoylpropyl-3-dimethyl-hydroxyethyl ammonium bromide), and DC-cholesterol (3b-N-(dimethylaminoethyl)carbamat-cholesterol) feature particular modifications of these domains.[88] One of the biggest problems of using lipid-based gene transfer includes the high toxicity at low specificity and sensitivity to serum components, precluding an efficient systemic use in clinical trials. Therefore, neutral or so-called ‘helper lipids’, such as the fusogenic phospholipid dioleoylphosphatidylethanolamine (DOPE) or the membrane component cholesterol, have been included in liposomal formulations to enhance transfection activity and stability. Cationic lipids have been successfully used to deliver the suicide gene Survivin threonine 34 to alanine (S-T34A) to treat colon cancer. After lipid-
based delivery, colon cancer cells expressed the particular protein, which then induced apoptosis, resulting in the growth inhibition of C-26 colon cancer cells in vitro.[89] Further, intraperitoneal administration of lipid-based S-T34A gene transfer inhibited growth of abdominal metastatic colon cancer and malignant ascites.[90] Further studies show the potential of cationic lipids as gene carriers. Since this system still faces limitations such as rapid clearance and generation of immune responses, lipid nanoemulsion, a dispersion of one immiscible liquid in another stabilized by emulsifying agent, was developed. These small 200 nm molecules, comprised of oil, water and surfactant, are considered to be superior to liposomes in scalability and stability. [94] Furthermore, SLNs were developed and represent the choice of delivery system for siRNA.[95] Intense development led to a variety of nanosystems useful for siRNA delivery, which are less cytotoxic and of increased therapeutic efficiency. Even nanoemulsions are intensively used for anticancer drug delivery; however, to date, only a few siRNA approaches have been reported.

Cationic lipids can be further used with modifications by adding ligands or fusogenic proteins, mainly composed of basic amino acids. One recent example is the peptide-based vector K(12)H(6)V(8)SSQHWSYKLDP (KHV-LHRH), which comprises four functional segments for the targeted delivery of luteinizing hormone-releasing hormone (LHRH) sequence. This particular vector specifically targets cancer cells expressing LHRH receptors, such as MCF-7 breast cancer cells or SKOV-3 ovarian cancer cells (cell line specific name). Due to these features, these peptides can be used to selectively target lipoplexes or polyplexes.[96]

4.1.2. Cationic polymers

Another class of nonviral DNA vectors consists of cationic polymers that are chemically diverse. This group includes not only the widely used poly (L-lysine) (PLL) and polyethylenimine (PEI), but also the less known carbohydrate-based polymers (chitosan, dextran, β-cyclodextrin), linear poly (amido-amine) (PAA), and others.[25] To change the physicochemical and biological properties, the structure and composition of polymers have been intensively studied. In general, PLL has poor transfection efficiency, as at physiological pH its amine groups are positively charged, leading to low endosomal buffering and lysis. Therefore, modified PLLs, such as PLL-PEG copolymers, were developed. Coating PLL with hydrophilic PEG minimizes nonspecific interaction with serum components and prolongs half-life during circulation.[115] PEI and its derivatives are the most studied polymeric materials and still considered as the ‘gold standard’ among the synthetic vectors. They are characterized by their linear and branched shape, which has undergone numerous modifications. Linear PEI, known as proton sponge, is commercially available (for example, jetPEI, in vivo PEI, etc.) and possesses high buffering capacity, which ensures endosomal release.[54] PELs, however, are highly cytotoxicity and, therefore, the PEI-PEG copolymer has been used to provide a more biocompatible nanoparticle. [116,117] The new-generation polymers are based on poly-[(2-dimethylamino)-ethyl methacrylate] (pDMAEMA), poly-arginine containing proteins, poly-(β-amino ester), and carbohydrate-based polymers, such as heparin and dextran. They are attractive as they are less toxic and also provide stability, biocompatibility, and biodegradability.[91,92]

Another group of chemical transfer vehicles are the dendrimers. They are highly branched polymer molecules carrying numerous functional groups on their surface, which allows for target gene attachment. The most common and promising dendrimers are polyamidoamines (PAMAMs) as they increased transfection efficiency by enhanced DNA uptake and endosomal escape. [89] These dendrimers effectively deliver pDNA as well as antisense oligomers and siRNA. A recent study demonstrated the successful achievement of RNA interference through use of the PAMAM dendrimer. The modified PAMAM dendrimer was able to effectively deliver sticky siRNAs, bearing complementary A overhangs, to a prostate cancer model in vitro and in vivo, leading to an anticancer effect.[93] Cationic polymers are promising for preclinical and clinical approaches but still need improved nuclear uptake.

4.1.3. Inorganic nanoparticles

Nanoparticles can be generally defined as subcellular objects <100 nm in size and can be classified based on their composition or physical properties. Additionally, they can be modified to induce tumor-targeting specificity. Nanoparticles made of silver or gold have been very promising as they are inert, non-immunogenic and capable of penetrating the blood–brain barrier.[97] Another very encouraging class of nanoparticles consists of the multi-walled carbon nanotubes [98] (cylindrical fullerenes) and magnetic nanoparticles, [99] as they carry plasmids that can target tumors. Further modifications, such as PEG attachment, have shown to promote a particularly intratumoral accumulation. The major drawback of using inorganic particles is their slow biological clearance and unwanted persistence. Therefore, studies on long-term safety, surface functionalization effect of type, size, and shape are needed to accelerate their clinical development.
4.2. Physical gene transfer

Physical gene transfer methods are based on delivering genetic material, such as naked DNA or RNA, by transient penetration into the cell membrane by mechanical, electrical, ultrasonic, or hydrodynamic energy, facilitating intracellular delivery of genetic material (Table 4). The physical methods have significantly improved the efficiency of nonviral gene delivery over the last decade.

4.2.1. Needle injection

It is long known that naked DNA or pDNA can be administered directly by intratumoral needle injection or applied systemically by intravenous injection. It is a simple and feasible transfer method, but it confers low transfection efficiency and transgene expression. However, needle injection is not very suitable for treatment of solid tumors, as the tumor’s high hydrostatic pressure within the tissue reduces persistence of the injected solution.[25] Therefore, it is only applicable for indications such as DNA vaccination.

4.2.2. Ballistic DNA transfer

This method, also known as gene gun or particle bombardment, employs accelerated DNA-coated gold, silver, or tungsten nanoparticles to force intracellular DNA transfer. The required speed is achieved by high-voltage electronic discharge or helium discharge. This method allows precise delivery of DNA, but the transfer is shallow. Until now, ballistic transfer has mainly been used in DNA vaccination gene therapies to treat acute lymphoblastic leukemia or skin cancer.[39,100]

4.2.3. Jet-injection

This needleless method is an alternative to inject genetic material. Mechanical compression is used to rapidly deliver genetic material by a small jet at high speed, which then penetrates the targeted tissue. Using this technology, a very small volume of naked or complexed nucleic acid can be introduced into the tissue. [101,102] The application of jet-injection can be used for any type of tissue, including tumors. By adjusting the parameters, intradermal gene transfer is also possible as an application for other tissues as soon as they can be reached for treatment. An increased pressure leads to an increased penetration depth, a larger transfer volume, and to a greater dispersion of the liquid jet. This approach has been applied for intratumoral and intramuscular gene delivery and is meanwhile in clinical testing for cancer gene therapy of malignant melanoma.[5]

4.2.4. Electroporation

Electroporation is a technique in which an electric field is applied to cells in order to increase the permeability of the cell membrane. The general procedure includes the injection of pDNA into targeted tissue and subsequent application of electric force for DNA entry. The transient pore formation occurs within 10 nanoseconds and allows genetic material to diffuse through the cellular membrane into the cell.[103,104] This process is reversible based on field strength and pulse duration. Generally, cancer cells require low field strength (<700 V/cm) with long pulse (milliseconds). The pulse can be adjusted to control the levels and duration of subsequent gene expression, allowing specific application. To date, more than 50 clinical trials employed electroporation for gene delivery, demonstrating bio-safety and tolerability (Table 1). Very recently, this method was used to deliver the antiangiogenic metargidin peptide (AMP) plasmid into melanoma cells, downregulating the α5β1 and αvβ3 integrins.[105] Electroporation has shown promise with high gene expression in specific targeted tissue but with limitations such as potential tissue damage and requirement of proper placement of electrodes by surgery.

4.2.5. Sonoporation

The noninvasive site-specific technique of sonoporation utilizes ultrasound waves to transiently permeabilize the cell membrane, allowing cellular uptake of genetic material. DNA is entrapped within microbubbles, which break apart and release shock waves nearby, causing transient pore formation of cell membrane. To achieve therapeutic levels of gene expression, the size of the microbubbles and the agent used in forming the bubbles are crucial. Gene delivery by sonoporation depends on many factors, including the frequency and intensity of ultrasound, the targeting ability of the microbubbles, DNA concentration, and duration of exposure.[106,107] The ultrasound-mediated gene delivery has the advantages of being capable of targeting internal organs without surgery and also of being noninvasive, efficient, targeted, and controlled. The local release at the site improves the safety and specificity of gene delivery for cancer treatment. Sonoporation has been used for the treatment of glioblastoma in vivo.[108] Nevertheless, ultrasound-mediated delivery has obstacles to overcome, as this delivery method provides less protection of genetic material against degradation and shear forces and low gene expression compared to other methods such as electroporation.
4.2.6. Magnetofection
To address the transient damage caused by invasive methods such as electroporation, magnetofection has been introduced. This delivery system uses a magnetic field to direct the delivery of genetic material to the desired target site of action. The DNA is attached to magnetic nanoparticles consisting of a biodegradable substance like iron oxide and coated with a polymer like PEI. These magnetic nanoparticles are then directed through a magnetic field generated by an external magnet, so that nanoparticles are pulled into the targeted cells for DNA uptake. This technique is noninvasive and can be precisely used for targeted gene therapy to increase gene expression within the targeted cells. The use of magnetic forces in the clinic is well perceived, as it is mainly used for imaging (MRI) and targeted chemotherapy within the tumors of patients.

5. Expert opinion
Preclinical and clinical development within the gene therapy field is dominated by therapeutics against cancer diseases. This is reflected by the fact that 64% of all gene therapy trials worldwide are aiming at the treatment of cancer. In this regard, the use of nonviral vectors (either as naked DNA/RNA or as complexed/encapsulated vector) is experiencing a productive revival during the last decade, so that meanwhile >17% of all gene therapy trials employ nonviral technologies. The current development can be explained by the tremendous improvements achieved for nonviral systems for more efficient and transcriptionally targeted and regulatable expression vectors with better transfer properties. This is associated with high flexibility in vector design and transgene capacity, access to plasmid_DNA with more defined vector topology (e.g. open circle; oc vs. supercoiled closed circular; ccc pdDNA) and improved safety in application.

Currently, most efforts are drawn toward significant size reduction of nonviral vectors by removal of unnecessary backbone sequences to enhance transgene expression, as well as vector delivery optimization. Focus in vector development is mainly on the generation of miniplasmid, minicircle, or dumbbell-shaped minimalistic vectors, paralleled by optimization of their production processes as important prerequisite for clinical applicability. Furthermore, successful combination of novel DNA-integration technologies (e.g. SB, PB, etc.) or of S/MARs with nonviral vectors, including plasmid or minicircle systems, significantly increased the duration and persistence of nonviral vectors and transgene expression in the target cells. This in fact increases the potential of nonviral vectors for prolonged transgene expression as of interest for immunostimulatory gene therapies to combat cancer. Here, particularly, engineering of T-cells with tumor-specific T-cell receptors (TCRs) or chimeric TCRs (CAR) is the major field of application for this type of vectors.

Other important advantages of nonviral vectors are the ease of production, scalability, and stability during storage, essential factors for broader clinical applicability. Nonviral vectors have therefore entered innovative fields in gene therapy, including gene suppression by RNAi approaches or the evolving field of anti-miR (AMO) only a little more than a decade from the first discovery of RNAi. These developments have been paralleled by the evolution of applicable delivery technologies (either chemical or physical), which ensure reproducible and more efficient transfer of the nonviral vectors. This is strongly supported by the fact, that liposomal as well as electroporation, gene-gun or jet-injection technologies have evolved into clinically used transfer technologies, demonstrating their safety and effectiveness. This field of gene delivery systems has been recently extended by the use of exosomes as novel cell-derived nanoparticles for encapsulation and cancer targeting of nonviral vectors.

Nonetheless, there are still severe limitations for nonviral vectors, which hinder the systemic application for the treatment of, e.g. cancer metastases. Here, further improvements are essential to better target their delivery in combination with sufficient vector stability to reach and to enter the right target cell/tissue for efficient therapeutic gene expression or target gene interference. In this context, the low transfer efficiencies and the ineffective nuclear uptake of nonviral vectors demand further research and development to achieve reproducibly high transfection rates as important prerequisites for their broader clinical application to treat cancer. In this regard, RNA therapeutics, such as chemically modified si/shRNA and miRNA, as well as DNA-oligonucleotides, such as aptamers and their combination as chimeras, hold promise for systemic applicability to treat the primary tumor and metastasis.

To implement novel technologies in cancer gene therapy, gene editing systems are currently under evaluation. Future studies will reveal whether gene editing systems such as sequence-specific zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALENs), or the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas (RNA-guide Cas9 nucleases) technology will gain more importance for gene therapy of cancer as novel approaches in gene correction therapy. These editing systems potentially allow the
sequence-specific correction of cellular genes in tumor cells (oncogenes or tumor suppressor genes) to interfere with the malignant phenotype of cancer cells. In this regard, ZFNs and TALENS can be targeted to specific genomic sequences by modification of their DNA-binding domains. By contrast, the CRISPR/Cas system can perform the gene modification via synthetic guide-RNA, which specifically binds to its target sequence. These technologies, however, are currently of interest for engineering of T-cells for antitumor targeting, rather than for direct gene correction in tumor cells.

In summary, recent advancements in size reduction of nonviral vectors in association with improved gene transfer and transgene expression efficiencies will enforce the clinical uses of nonviral gene therapy. Great potential lies in the fusion of small vectors, such as minicircle and dumbbell-shaped vectors with components, which mediate vector integration into the genome (SB, PB, S/MAR, etc.) and which permit prolonged transgene expression for improved therapeutic efficacy. Further developments for better targeting of nonviral vectors will promote their systemic applicability, which is of particular importance to treat cancer metastases. This is achieved by stabilization of therapeutic nucleic acids by chemical modification to prevent degradation and/or clearance, and by combination of therapeutic nucleic acids (DNA, si/shRNA, miRNA, etc.) with nanoparticles, peptide mimetics, aptamers, etc., mediating tumor targeting and increased cellular entry.

In fact, the clinical trials performed with nonviral vectors demonstrate great therapeutic potential either as sole gene therapy or as adjuvant therapy in combination with conventional therapies such as chemo- or radiotherapy.

References
Papers of special note have been highlighted as either of interest (*) or of considerable interest (++) to readers.


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Declaration of interest
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- This article is one representative example for clinical use of antisense ODNs targeting c-raf for treating cancer patients.


- This clinical trial shows the promising gene suppression approach of using siRNA targeting protein kinase PKN3 for the treatment of cancer patients.


- This is the first report of successful application of ODN decoy gene suppression therapy in patients, which is based on specific decoy of transcription factor activity.


- This study demonstrates the successful use of xenogenic naked anti-humanp53 DNA vaccine to immunize immune competent mice, which efficiently provokes immune responses against the human p53, circumventing the problems with initially low immunogenicity of p53. The stimulation of anti-p53–antibody production and specific CD8+ T-cells in association with potent antitumor effects has great potential to treat metastasizing disease.


- This report demonstrates the application of sleeping beauty transposon system to generate genetically modify T cells for clinical trials.


36. Vyazunova I, Maklakova VI, Berman S, et al. Sleeping Beauty forward genetic screen identifies new genes and


• This report supports the advantages and potential of S/MAR based replicating minicircle in gene therapy.


• This report supports the advantages and potential of using minicircle-based vectors for gene therapy.


This study supports the efficient use of dumbbell-shaped minimalistic vectors for improved transgene expression, as shown for TNF-alpha in vitro and in vivo.


This report describes the development of AS-ODNs in vitro and in vivo as well as their pre-clinical and clinical development in therapy of human gliomas.


This study highlights the therapeutic potential of aptamers for targeted cancer gene therapy.


In this clinical Phase I study successful systemic delivery of lipid nanoparticles-formulated siRNA targeting VEGF and KSP is demonstrated in cancer patient suffering from liver metastases, leading to antitumoral activity at hepatic and extrahepatic sites of disease, including a complete response.


• This report demonstrates the potential targeted therapy for melanoma after gene electrotransfer of a AMEP plasmid, as inhibition of proliferation, migration and invasion were observed.


