Improving therapeutic potential of non-viral minimized DNA vectors

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Abstract

The tragic deaths of three patients in a recent AAV-based X-linked myotubular myopathy clinical trial highlight once again the pressing need for safe and reliable gene delivery vectors. Non-viral minimized DNA vectors offer one possible way to meet this need. Recent pre-clinical results with minimized DNA vectors have yielded promising outcomes in cancer therapy, stem cell therapy, stem cell reprogramming, and other uses. Broad clinical use of these vectors, however, remains to be realized. Further advances in vector design and production are ongoing. An intriguing and promising potential development results from manipulation of the specific shape of non-viral minimized DNA vectors. By improving cellular uptake and biodistribution specificity, this approach could impact gene therapy, DNA nanotechnology, and personalized medicine.

INTRODUCTION

In 2017, we wrote a comprehensive review of the history, key developments, specialized uses, and broad outlook for non-viral minimized DNA vectors as therapeutics, and, in some cases, as critical enablers of other cell-based therapies (e.g., stem cell reprogramming) [1]. We described in detail the many advantages minimized DNA vectors offer. In brief, removal of immunogenic bacterial sequences and antibiotic resistance genes from plasmids allowed for a dramatic reduction in vector length and led to the emergence of a new generation of non-viral gene delivery vectors (minimized DNA vectors). Minimized DNA vectors do not integrate into the genome and encode only therapeutic sequences. Reduced vector length is one of many factors that is likely to account for the observed increased levels and duration of gene expression compared to other non-viral vectors, particularly plasmids (some comparisons of vector systems are summarized in Table 1) [2–6].

There are several types of non-viral minimized DNA vectors in pre-clinical use (reviewed in [1]). Here, we will highlight recent advances for minicircles [1,7,8] and minivectors [1,9,10]. Several different methods exist for the production of these vectors [7,8,11], but common to most is the use of bacteria to propagate plasmids. Bacteria are induced to express enzymes that catalyze recombination of these parental plasmids. This reaction excises the bacterial propagation sequences into a separate molecule (the ‘miniplasmid’) that can be removed either by endonuclease-mediated degradation in the bacteria [12] or by size-exclusion chromatography [11,13]. Complete removal of unrecombined parent plasmid, miniplasmid, immunogenic endotoxin, and bacterial genomic DNA is laborious and time-consuming, yet essential. Recently, a production method was developed that relies upon a multiplex PCR protocol for minicircle formation [6]. This method circumvents the use of bacteria, eliminating the need for removal of bacterial contaminants and, thus, can be completed in
hours versus days. The product vectors, dubbed ‘bacteria-free minicircles,’ could be a useful tool for gene therapy, but production scale-up may still be an issue [6].

In common, minicircles and minivectors are double-stranded, circular, supercoiled DNA vectors encoding therapeutic sequences. One key difference between the two is that minivectors employ a more rigorous purification method that takes advantage of the small size of the minivectors generated, allowing for complete removal of the larger miniplasmid contaminant. Additional advantages include increased negative supercoiling and the ability to generate vectors as small as a few hundred base pairs [9,10].

The reduced size of minimized DNA vectors allows for the delivery of many more therapeutic molecules per given unit of mass. Therefore, much less mass of DNA is required to deliver an equivalent number of molecules. Minimized DNA vectors may thus be advantageous for delivering higher doses of a potential therapy without evidence of the cytotoxic effects that prohibit the use of higher doses of plasmids. Less mass of vector also means less delivery vehicle and thus reduction of another potential source of toxicity. The decreased toxicity and decreased immunogenicity of minimized DNA vectors, and especially of minivectors, may help mitigate some of the adverse effects observed in gene therapy clinical trials, such as in the recent X-linked myotubular myopathy clinical trials that used adeno-associated virus (AAV) [14–16].

Exciting pre-clinical work with non-viral minimized DNA vectors has continued since our last review in 2017 [1], bringing the field closer to realizing the hope of wide-spread clinical success. In this brief update, we summarize these new developments, concentrating on two key applications where progress has been most impressive—cancer therapy and stem cell therapy. We also present a new idea stemming from an improved understanding of DNA structure. With support from computational simulation data to illustrate the feasibility of the approach, we demonstrate that it may be possible to manipulate the shape of DNA vectors for selective tissue or cell targeting, and/or increased cellular uptake.

**USING MINIMIZED DNA VECTORS FOR CANCER THERAPY**

To date, the field that has probably benefited most from minimized DNA vector technology is that of cancer therapy, particularly in the development of chimeric antigen receptor T cells (reviewed in [17]). Chimeric antigen receptors (CARs), so named because they artificially fuse antigen-binding domains to specific cell-activating domains [18], have brought the gene therapy field some of its first clinical and commercial achievements (e.g., Kymriah®, Yescarta®). Although CAR T cell therapy has been successful, particularly for hematological malignancies [19], improvements are still needed. The therapy can be immunogenic and the protocol for developing and delivering the T cells is expensive, complicated, and takes several weeks. Non-viral minimized DNA vectors could replace the viral vectors used to engineer autologous (or allogeneic) CAR T cells [20], resulting in cheaper, faster, and safer production. Indeed, minicircles encoding a CD44-CAR have been electroporated into T cells to engineer them against hepatocellular carcinoma. The resultant CD44-CAR T cells resembled normal T cells in cytokine profile and phenotype, specifically lysed CD44+ cell lines and not CD44- cell lines, and suppressed tumor growth in vivo.
compared to controls [21]. This result was important as it demonstrated the efficacy of minicircle-generated CAR T cells against a solid tumor, which is more challenging to treat than the diffuse lymphomas treated previously [19].

Similar breakthroughs of minicircle-generated CAR T cells have also been reported for prostate cancer [22] and colorectal cancer [23]. Cheng et al. (2019) successfully generated anti-CD19 CAR T cells via electroporation with minicircles generated using the bacteria-free production method described above [6]. The resultant CAR T cells decreased tumor burden in mice with at least the same efficacy as lentiviral-generated CAR T cells carrying the same anti-CD19 CAR genes [6]. Furthermore, Batchu et al. (2019) engineered CAR natural killer (NK) cells capable of killing pancreatic cancer cells in vitro using a combination of minicircles encoding a mesothelin CAR and Sleeping Beauty transposition [3]. CAR T cell therapy requires the ex vivo modification of autologous T cells from each individual. In contrast NK cells, because their cytolytic activity is antigen-independent, can be taken from healthy donors and engineered in advance of therapy. This process creates an off-the-shelf product that saves both time and money. Of all the minicircle-based applications currently in development, use of the non-viral Sleeping Beauty transposon system for the safe and reasonably effective generation of CAR T cells is probably the closest to achieving clinical efficacy [20,24].

Various other minicircle-based strategies have emerged for breast cancer [4,25], brain cancer [5], ovarian cancer [26], nasopharyngeal carcinoma [27] and other applications (Table 2). Kanada et al. (2019) developed a method that uses microvesicles to deliver minicircles encoding prodrug converting enzymes [4]. The expressed enzymes convert co-delivered prodrugs into cytotoxic agents that kill tumor cells. Minicircles were also combined with calcium phosphate nanoneedles for ovarian cancer [26] and others used liposome-templated hydrogel nanoparticles to deliver both Cas9 protein and minicircles encoding guide RNA intravenously to tumor cells in the brain [5]. When polo-like kinase 1 was targeted for inhibition in brain cancer cells, tumor burden was decreased and survival of mice increased [5]. Finally, in Wu et al., (2017) nasopharyngeal carcinoma cells were targeted by way of a commonly expressed Epstein–Barr virus antigen (EBNA1) that selectively triggers the expression of a microRNA that inhibits nasopharyngeal carcinoma cell growth and metastasis [27].

Minimized DNA vectors have had a broad range of applicability throughout the cancer field and their use has also helped make headway in other disease areas, such as retinal disorders [28,29], rheumatoid arthritis [30], Parkinson’s disease [31], and inborn errors of metabolism [32]. They have even been used for the endogenous production of biologics [33]. Their impact has also been felt in the areas of, among others, anti-viral treatments [34], vaccination [35], and regenerative medicine [36,37].

**USING MINIMIZED DNA VECTORS FOR STEM CELL THERAPY & STEM CELL REPROGRAMMING**

Regenerative medicine uses autologous (or allogeneic) stem cells for the repair or replacement of damaged or diseased tissue. A major limitation to this approach has been
associated with the use of integrating viruses, such as retroviruses or lentiviruses, to deliver the appropriate enabling therapeutic genes to stem cells. The potential for insertional mutagenesis is high, which could lead to disastrous downstream consequences. Minimized DNA vectors have been tested as a replacement for viral vectors to mitigate these safety issues. Several varieties of stem cells have been successfully manipulated using minicircles, including neural stem cells [38,39], mesenchymal stem cells [40,41], skeletal myogenic progenitors [42], and hematopoietic stem cells [43]. Most frequently this work has been done in mouse and human cells, but canine and equine cells have also been used [40].

Minicircles have further been used to enable stem cell reprogramming, which refers to the process of reverting mature, differentiated cells into pluripotent stem cells capable of expanding indefinitely and differentiating into all other cell types in the body (called induced pluripotent stem cells or iPSCs). iPSCs are classically produced using somatic cells transduced with integrating viruses that carry genes for the cellular reprogramming factors needed to induce reversal of the developmental state [44]. As with the other types of stem cells described above, however, the use of integrating viruses renders iPSCs produced in this manner inappropriate for clinical translation. Indeed, chimeric mice generated from iPSCs produced with virus and then injected into blastocysts formed tumors [45].

The persistent safety issues surrounding integrating viruses have spurred research into alternative approaches (reviewed in [46]). In addition to minimized DNA vectors, other non-viral [46,47] methods for stem cell reprogramming include plasmids, mRNA [48,49], microRNA [50,51], and transposon systems, such as Sleeping Beauty (the components of which can be encoded on either plasmids or minimized vectors) for the safer genomic integration of DNA sequences. The use of non-integrating viruses such as Sendai virus [52], adenovirus [53], AAV [54], and measles virus [55], has also been explored. Fortunately, insertional mutagenesis is not required for the production of iPSCs [53], and thus it is feasible to use non-integrating vectors. Even with most non-integrating viruses, however, there is still a small chance for genomic integration and even gene expression from integrated vector DNA beyond the point at which reprogramming has taken place [56]. Other difficulties with viruses include immunogenicity, limits on the size of the therapeutic insert, and variable tropism, which makes it so that some viral systems will not work well with some cell types. Other approaches to stem cell reprogramming forgo the use of genetic material entirely (thoroughly reviewed in [57]). These methods, however, are still very technically challenging and often result in a low yield of usable cells.

Non-viral minimized DNA gene therapy approaches provide a valuable option for stem cell reprogramming as they are safer and less complex to use. For example, minicircles expressing bone morphogenetic protein 2 and transforming growth factor beta 3 were used in a strategy for cartilage treatment and regeneration. Mesenchymal stem cell-like, human iPS-derived outgrowth cells transfected with minicircles successfully differentiated into cells of the chondrogenic lineage. Chondrogenic pellets derived from these cells also corrected defects in a rat osteochondral defect model [58]. In an interesting development for cancer treatment, minicircles were used to reprogram murine melanoma cells. The reprogrammed cancer cells were less malignant than non-reprogrammed cancer cells, as
evidenced by a smaller proportion of cells in S-phase and by the formation of smaller tumors in mice [59].

It is important to keep pushing stem cell/iPSC research forward because these cells are critically needed for drug screening, organ and tissue generation, and disease modeling. Enabling the study of the patient-specific basis of disease also further advances personalized medicine. While not without challenges [42], minimized DNA vectors should continue to advance this field.

THE DIFFICULTY OF TRANSLATING NON-VIRAL MINIMIZED DNA VECTORS TO THE CLINIC

Despite the encouraging successes described above, significant hurdles have slowed the advancement of minimized DNA vectors into the clinic. One hurdle has been the achievement of high quality yet cost-effective scale-up of the vectors. Fortunately, gains are being made in improving vector yields and in minimizing contaminants [60–62], which will ultimately lower the cost of production (briefly reviewed in [63]).

Viral vectors are generally more efficient than non-viral vectors at delivering a genetic payload. Perhaps reflecting this difference, nearly two-thirds of gene therapy clinical trials are based on viral rather than non-viral methods [24]. Transient gene expression from non-viral vectors is another hurdle. For example plasmids, which thus far have been the most commonly employed non-viral vector in clinical trials [1], are prone to silencing [64–66] and have generally failed to afford long-enough lasting benefit in patients [67,68]. Minimized DNA vectors are much less susceptible to transgene silencing than plasmids and are capable of producing long-lasting gene expression [2–6]. Substituting plasmids with minimized DNA vectors should provide the benefit of stable and pro-longed gene expression.

Physical or chemical means are required to carry non-viral DNA vectors into cells [1,69–74]. Once inside the cell, vectors must also enter the nucleus to express the encoded therapeutic cargo. Nuclear trafficking of DNA, however, is a complex and not yet fully understood process [75]. In the cell cytoplasm, DNA associates with proteins to facilitate migration toward the microtubule organizing center and the nuclear envelope [76]. If the DNA vector delivered is large, organelles and translation machinery in the cytoplasm prevent free diffusion inside the cells [64,77] and across the nuclear membrane pore channel [78]. DNA ≥2,000 bp is unable to diffuse into the perinuclear space [79]. In addition, the inner diameter of the nuclear pore complex is ~40–42.5 nm [75,80–82]. Hence, small and compact DNA particles are more likely than plasmids to successfully traverse the cell, avoid degradation, and diffuse through the nuclear pore. Minivectors, for example, are typically shorter than 2,000 bp in length and can be as small as ~40 nm in diameter [83], facilitating passage through cell and nuclear membranes.
DESIGN OF NANOPARTICLES FOR IMPROVED CELLULAR UPTAKE

The field of nanotechnology takes advantage of the benefits provided by nanometer-sized particles [84], and the advances made in this field could potentially be used to inform the design of the next generation of minimized DNA vectors. Nanoparticle size is important not only for cellular internalization but also for retention [85,86], as persistence can have major implications for therapeutic delivery and gene expression. Cancer-targeting nanoparticles less than 100 nm in diameter freely diffuse through tumor pores and accumulate within tumors [86,87]. Based on thermodynamic modeling studies of ligand-coated nanoparticles, the optimal particle size for cellular uptake should be between 25–30 nm [88]. Maximum in vitro uptake of polystyrene and gold particles in cultured HeLa cells was achieved when particles were between 25–42 nm [89] and 50 nm [90], respectively. 50 nm was also the most effective size for uptake of silver nanoparticles by red blood cells [91]. In 3-D cultures, fluorescently labeled carboxylic acid-modified nanoparticle beads ≥100 nm were restricted from cellular uptake, whereas particles ≤40 nm were not [92]. In vivo, drug-silica nanoconjugate particles of 50 nm display maximum tissue retention and deep tumor penetration [93]. Gold nanoparticles of 15 and 50 nm are even able to effectively cross the blood–brain barrier [94] and accumulate faster in tumors than particles ≥60 nm. For larger tumor volumes, however, the larger nanoparticles accumulated better [95]. Whereas smaller particles are generally more effective, particles that are too small are not. Inert nanoparticles with diameter <10 nm are quickly eliminated by the kidneys [96,97]. RNA nanoparticles of <5 nm are also promptly cleared after injection in mice [98]. Even variations as small as 2 nm may change biodistribution [97].

Nanoparticle shape also influences cellular and nuclear uptake [99,100]. Filamentous particles are more effective at cellular uptake than spherical systems [86]. Nanoparticles with sharp edges escape endosomes faster, avoid exocytosis, and persist longer inside cells than those with rounded edges [101]. Similarly, gold nanotriangles are more readily taken up by cells than nanorods or nanostars [100]. Structural differences of polyethyleneimine/DNA nanoparticles dictate their cellular uptake mechanism (macropinocytosis-mediated versus clathrin-mediated endocytosis), thus resulting in different transfection and gene expression levels [77]. In vivo, particle shape affects venous circulation, biodistribution, cellular uptake [102], and influences tumor penetration [103]. Uptake of nanoparticles by cells is dependent not only on size and shape, but also surface area, flexibility, and charge [97,104,105]. Altering these parameters targets nanoparticles to different tumors, tissues, and cell types [96].

Nanotechnology has the potential to address chronic diseases through controlled, site-specific delivery of precise medicine [84,106–109], as well as through the development of multimodality agents with both imaging and therapeutic capabilities [85,108,110,111]. Nanoparticles have great potential for the treatment of cancer and other diseases [85,96]. Obstacles still remain, however. The materials that make up some nanoparticles contain heavy metals, which may be toxic [91,109,110,112], and systemic delivery of nanoparticles is difficult [106].
DESIGN OF DNA MINIVECTOR NANOPARTICLES

Since the concept was first proposed in the 1980s, significant progress has been made in constructing nanostructures made of DNA [113,114]. DNA is remarkably stable [115–117] and the strict rules for pairing between bases allow for the rational design of increasingly complex DNA nanostructures [118,119]. Current DNA nanotechnology applications include construction of structural lattices, scaffolds, molecular machines, bio-sensors, and targeted drug delivery systems [113,120–122]. The properties of DNA make it suitable for the construction of a nearly limitless choice of nanostructures that can be further controlled and modified by a variety of DNA-acting enzymes [120,123,124].

Although assembling DNA into complex nanostructures holds promise for clinical applications (reviewed in [120]), the process of making these structures is far from straightforward. First, a large number of DNA fragment components are typically required to build these structures, which increases the likelihood of incorrect assembly. Second, annealing products correctly requires very long folding times, limiting throughput. Third, products need to be purified, but protocols for purification have not been fully optimized. Fourth, the procedures required for annealing and purification are difficult to scale up, resulting in low yield. Finally, even though composed of DNA, none of these structures are themselves the ‘active’ therapeutic component, but rather serve as the carrier for, or foundation of, delivery or construction of other nanoparticles [125–128]. Indeed, many of the breakthroughs in nanotechnology for gene therapy are based upon the design of synthetic nanoparticles as delivery vehicles for nucleic acid payloads into the cell and none have yet focused on modification of the therapeutic-encoding DNA vector itself.

With diameters of around 40 nm [83], supercoiled minivectors can be made small enough such that their diameter is within a nanoparticle size range [10,83]. Furthermore, when complexed with delivery vehicle, for example, poly-L-lysine-polyethylene glycol, minivectors are highly homogenous, monodisperse, and adopt a needle-shaped conformation; comparatively, plasmids are not nanoparticle-sized and adopt highly heterogeneous shapes (Figure 1). These parameters are all important for cellular and nuclear entry.

Could DNA minivectors be both the nanoparticle and the genetic payload? By using DNA supercoiling and adding ‘bend site’ sequences, it seems possible. Certain DNA sequences are much more flexible than others [129,130]. Additionally, because single-stranded DNA is more flexible than double-stranded DNA [131], disruptions to base pairing can generate hyperflexible hinges to facilitate bending [132–135]. The propensity for base pair disruption in supercoiled DNA is also sequence-dependent [136]. Based on these principles, certain sequences are more likely to bend, either because they are intrinsically more flexible, or because of disruptions to base pairing [Fogg et al., 2020, submitted]. The placement of bend sites could influence the 3-D structure of supercoiled DNA molecules. Therefore, by modifying the DNA sequence, we hypothesize that it should be possible to manipulate minivector DNA shape with supercoiling.
We demonstrated the feasibility of this approach by simulating, using established computational models [137–140], the effect of engineering three bend sites in a supercoiled 336 bp minivector and predicted that this should cause the DNA to adopt three-lobed shapes [141]. Because the high compaction of rod-shaped minivectors may offer improved cellular uptake, we reasoned that introducing a bend site diametrically (180°) opposite another bend site in the 336 bp minivector could result in a strong mechanical correlation between the two sequences. If correct, the two sites should then facilitate bending at the two apices of the rod to stabilize the rod-shaped conformation (Figure 2A).

Using coarse-grained molecular dynamics simulations with oxDNA [142], we found that the unmodified (no added bend site) negatively supercoiled minivector sequence formed a rod-shaped conformation 30% of the time simulated across 10 independent simulations (Figure 2B). This prediction is in good agreement with the fraction of rod-shaped conformations observed directly in this minivector [83]. When the modified minivector sequence containing one bend site opposite the other bend site was simulated, the fraction of rod-shaped conformations observed increased to 61%. We observed the predicted bend sites localizing to the apices of these rod-shaped conformations as well as base pair disruption accompanying the bend sites (Figure 2B). In the simulations, once the rod-shaped conformation formed with bend sites at the apices, it was typically stable for the remainder of the simulation. Minivectors with the unmodified sequence (with a single bend site) fluctuated among multiple different conformations. Simulations, therefore, predict that we can use circularity, DNA supercoiling, and sequence to enrich for certain nanoparticle shapes.

Simulations suggest that it may be possible to design at least two different novel DNA shapes (rod-shaped and three-lobed conformations). These two shapes have potential for targeted therapy. Lung tissue selectively accumulates star-shaped over spherical gold nanoparticles [143]. Rod-shaped are more amenable to cellular transfection in clinically relevant breast cancer cell lines compared to spherical polystyrene nanoparticles [144]; the authors of this study [144] speculated that the increased surface area of rods allows for more contact with the cell membrane. Nanoparticles with higher aspect ratios (i.e., much longer than they are wide, as in the rod-shape) also seem to be more effective at avoiding clearance through phagocytosis—an important pharmacokinetic characteristic [145–147]. Specific shapes of non-viral minimized DNA vectors could thus exhibit tissue specificity and improved cellular uptake, with implications for targeted therapies.

CONCLUSIONS

The recent pre-clinical results summarized here showcase the benefits of using minimized DNA vectors for therapeutic purposes. There is still plenty of room for improvement in vector design and in advancing non-viral minimized DNA vectors to the clinic. Difficulties remain in production scale-up and in getting DNA vectors into cells efficiently. One avenue for improvement takes advantage of two important features of nanotechnology: particle size and shape. The smallest minimized DNA vectors (minivectors) fall within the range of ideal sizes for cellular uptake. Strategically placed bend sites in supercoiled minivectors may
enable specific nanoparticle conformations that could one day prove beneficial for gene therapy and targeted nanomedicine.

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FIGURE 1. Transmission electron micrographs of three DNA vector sizes. Poly-L-lysine-polyethylene glycol and DNA were complexed at a nitrogen:phosphate ratio of 2:1. Z-average (a measure of particle size), ζ-potentials (a measure of the degree of electrostatic repulsion between adjacent particles), and polydispersity index (PDI, a measure of the amount of variability in the particle size distribution) values were determined using dynamic light scattering using a Malvern Zetasizer Nano (data courtesy of Dr Jin Wang, Dr Fude Feng, and Dr. Daniel J Catanese, Jr.).
FIGURE 2. Generating custom minimized DNA vector shapes.
(A) Schematic representation of the predicted effect of adding bend sites. Bend sites (red) are flexible, which should localize them to superhelical apices with supercoiling. (B) Representative image from the coarse-grained simulations showing the rod-shaped conformation, (the conformation observed most frequently with two bend sites), and summary of how frequently rod-shaped conformations were observed during the simulations, and of these rod-shaped conformations, what percent had the bend site(s) localized to the apices.

| Sequence         | Observed rod-shaped conformation (%) | Bend sequences(s) located at rod apices (%) |
|------------------|--------------------------------------|--------------------------------------------|
| One bend site    | 30                                   | 54                                         |
| (control)        |                                      |                                            |
| Two bend sites   | 61                                   | 74                                         |

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Outcomes of studies comparing non-viral minimized DNA vectors to other vector systems.

| Vectors used | Vector length | Sequence encoded | Transfection method | Outcomes |
|--------------|---------------|------------------|---------------------|----------|
| Minicircle   | 3.881         | Firefly luciferase| Sequence-defined oligoamino amides/cationic polymer | Compact, rod-shaped polyplexes were 65–100 nm using plasmid and 35–40 nm using minicircle; all formulations of minicircle polyplexes lacked cell cycle dependence. Mini-circle transfected 3-fold more than equal moles of plasmid. Combined, tyrosine trimer integration, combination polyplexes, and use of minicircle increased gene expression ~200-fold over an equal mass of plasmid. |
| Minicircle   | 4.573         | Mesothelin CAR   | Electroporation     | CAR expression, IFNγ and granzyme B secretion, and specific lysis of pancreatic cancer cell lines was significantly increased in NK cells electroporated with minicircle over plasmid. Use of minicircle resulted in increased NK cell viability after electroporation. |
| Minicircle   | 3.700*        | Firefly luciferase| Microvesicles/cationic lipid | Equal moles of minicircle resulted in prolonged transgene expression in breast cancer cells. Minicircles loaded into microvesicles twice as efficiently as equal moles of plasmid but resulted in a peak bioluminescent signal 14 times higher than in cells treated with microvesicles containing plasmid. Microvesicles loaded with minicircles encoding TK/NTR led to greater activity of produg converting enzymes over microvesicles with equal mass of plasmid. |
| Minicircle   | 4.000*        | TK/NTR           | Electroporation     | Mini-circle transfected twice as efficiently as an equal mass of plasmid. Minicircle had higher capacity to deliver to primary retinal cells and rat retinas than equal mass of plasmid. |
| Minicircle   | 7.900*        | eGFP             | Cationic lipid      | Minicircle transfected twice as efficiently as an equal mass of plasmid. Minicircle had higher capacity to deliver to primary retinal cells and rat retinas than equal mass of plasmid. |
| Minicircle   | 364           | Guide RNA (to inhibit PLK1) | LHNPs | Cas9 protein co-delivered in LHNPs with minicircles decreased PLK1 expression more than Cas9 protein co-delivered with plasmid or minicircle co-delivered with Cas9 DNA in vitro. |
| Minicircle   | 8,318         | 2nd gen. anti-CD19 CAR | LHNPs | LHNPs were comparably effective in delivering Cas9 to liver and brain. |
| Minicircle   | 2,257         | eGFP             | Cationic lipid      | Minicircle transfected twice as efficiently as an equal mass of plasmid. Minicircle had higher capacity to deliver to primary retinal cells and rat retinas than equal mass of plasmid. |
| Minicircle   | 3,487         | eGFP             | Cationic lipid      | Minicircle GFP expression in retinal cells was maintained for 7 days while GFP expression from an equal mass of plasmid was lost by day 7. Gene delivery to retinal cells in vivo using AA V or minicircle encoding rhodopsin was comparable to that from plasmid vectors. Cells modified ex vivo with AA V or minicircles encoding rhodopsin reconstructed functional retinal tissue and supported vision function in blind mice. |
| Minicircle   | 5,541         | GFP               | Cationic lipid      | Minicircle GFP expression in retinal cells was maintained for 7 days while GFP expression from an equal mass of plasmid was lost by day 7. Gene delivery to retinal cells in vivo using AA V or minicircle encoding rhodopsin was comparable to that from plasmid vectors. Cells modified ex vivo with AA V or minicircles encoding rhodopsin reconstructed functional retinal tissue and supported vision function in blind mice. |
| Minicircle   | 2,500         | Rhodopsin        | Cationic lipid      | Plasmids encoding genes with or without Cre recombinase were transfected into Salmonella as a platform for oral DNA vaccination against Newcastle disease virus in poultry. Plasmid containing Cre recombinase allowed for the in vivo generation of minicircle encoding either eGFP or HN. Chickens orally inoculated with Salmonella transfected with Cre/eGFP-containing plasmid contained significantly more eGFP in liver than plasmid without Cre. Chickens that received Cre/His CR inoculation were protected against challenge with NDV significantly more than chickens inoculated with Salmonella containing HN plasmid alone. |
| Minicircle   | 7,722         | eGFP             | Cationic lipid      | Plasmids encoding genes with or without Cre recombinase were transfected into Salmonella as a platform for oral DNA vaccination against Newcastle disease virus in poultry. Plasmid containing Cre recombinase allowed for the in vivo generation of minicircle encoding either eGFP or HN. Chickens orally inoculated with Salmonella transfected with Cre/eGFP-containing plasmid contained significantly more eGFP in liver than plasmid without Cre. Chickens that received Cre/His CR inoculation were protected against challenge with NDV significantly more than chickens inoculated with Salmonella containing HN plasmid alone. |
| Minicircle   | 9,668         | eGFP/Cre         | Cationic lipid      | Plasmids encoding genes with or without Cre recombinase were transfected into Salmonella as a platform for oral DNA vaccination against Newcastle disease virus in poultry. Plasmid containing Cre recombinase allowed for the in vivo generation of minicircle encoding either eGFP or HN. Chickens orally inoculated with Salmonella transfected with Cre/eGFP-containing plasmid contained significantly more eGFP in liver than plasmid without Cre. Chickens that received Cre/His CR inoculation were protected against challenge with NDV significantly more than chickens inoculated with Salmonella containing HN plasmid alone. |
| Minicircle   | 8,783         | recombinase      | Cationic lipid      | Plasmids encoding genes with or without Cre recombinase were transfected into Salmonella as a platform for oral DNA vaccination against Newcastle disease virus in poultry. Plasmid containing Cre recombinase allowed for the in vivo generation of minicircle encoding either eGFP or HN. Chickens orally inoculated with Salmonella transfected with Cre/eGFP-containing plasmid contained significantly more eGFP in liver than plasmid without Cre. Chickens that received Cre/His CR inoculation were protected against challenge with NDV significantly more than chickens inoculated with Salmonella containing HN plasmid alone. |
| Minicircle   | 10,684        | recombinase      | Cationic lipid      | Plasmids encoding genes with or without Cre recombinase were transfected into Salmonella as a platform for oral DNA vaccination against Newcastle disease virus in poultry. Plasmid containing Cre recombinase allowed for the in vivo generation of minicircle encoding either eGFP or HN. Chickens orally inoculated with Salmonella transfected with Cre/eGFP-containing plasmid contained significantly more eGFP in liver than plasmid without Cre. Chickens that received Cre/His CR inoculation were protected against challenge with NDV significantly more than chickens inoculated with Salmonella containing HN plasmid alone. |

| Ref. | |
|------|---|
| [2]  | |
| [3]  | |
| [4]  | |
| [5]  | |
| [6]  | |
| [9]  | |
| [10] | |
| [28] | |
| [29] | |
| [35] | |
| Vector used | Sequence encoded | Vector length | Transfection method | Ref. |
|------------|-----------------|---------------|---------------------|------|
| Minicircle | Bcl-2 | 1,715 | Cationic lipid | [41] |
| Plasmid | GFP | 3,088 | Cationic lipid | [38] |
| Minicircle | GFP/Sox9 | 4,618 | Electropermeabilization/microinjection | [40] |
| Plasmid | VEGF | 1,715 | Cationic lipid | [41] |
| Minicircle | hPAX7/GFP | 4,129 | Nucleofection | [42] |
| Plasmid | Venus fluorescent protein | 3,400 | Nucleofection | [43] |
| Minicircle | SB100X transposase | 6,100 | Nucleofection | [43] |

*Italicized values are estimated lengths provided by the authors of those studies.*

1. This study focused on DNA vector length.
2. This study sought to use plasmids harboring Cre recombinase and the gene of interest for the in vivo production of minicircles carrying the gene of interest without Cre recombinase by using Cre expression to recombine the originally transfected plasmid, effectively amounting to the delivery of both plasmid and minicircle to the Salmonella cells receiving plasmid encoding Cre recombinase.
| Ref. | Vector length | Vector | Transfection method | Outcomes |
|------|--------------|--------|---------------------|----------|
| [21] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44 CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [22] | NR           | NR     | Electroporation     | Unlike normal T cells, minicircle-generated PSCA CAR T cells had high cytokine secretion, strong antitumor effects, and improved survival of mice. |
| [23] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [24] | NR           | NR     | Electroporation     | Unlike normal T cells, minicircle-generated PSCA CAR T cells had high cytokine secretion, strong antitumor effects, and improved survival of mice. |
| [25] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [26] | NR           | NR     | Electroporation     | Unlike normal T cells, minicircle-generated PSCA CAR T cells had high cytokine secretion, strong antitumor effects, and improved survival of mice. |
| [27] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [28] | NR           | NR     | Electroporation     | Unlike normal T cells, minicircle-generated PSCA CAR T cells had high cytokine secretion, strong antitumor effects, and improved survival of mice. |
| [29] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [30] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [31] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [32] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [33] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [34] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [35] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [36] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [37] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [38] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [39] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [40] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [41] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [42] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [43] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [44] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [45] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [46] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [47] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [48] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [49] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [50] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [51] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [52] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [53] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [54] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [55] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [56] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [57] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [58] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |
| [59] | NR           | NR     | Electroporation     | Minicircle-generated anti-CD44-CAR T cells expressed CAR molecules with strong hepatocellular carcinoma tumor suppression activity in vivo and overcame tumor xenograft outgrowth barriers in mice. |

**TABLE 2**

Studies using minicircles for the development of novel therapies.
| Sequence encoded | Vector length | Transfection method | Outcomes |
|------------------|---------------|---------------------|----------|
| cells, however, still displayed the characteristics of cancer stem cells and formed smaller, less aggressive tumors than the parental cell line. |

*Italicized values are estimated lengths provided by the authors of those studies.*

Becl-2: B-cell lymphoma 2; BMP2: Bone morphogenic protein 2; CAR: Chimeric antigen receptor; CBS: Cystathionine β synthase; CD3: Cluster of differentiation 3; CD44: Cluster of differentiation 44; CD8: Cluster of differentiation 8; EpCAM: Epithelial cell adhesion molecule; FLS: Fibroblast-like synoviocytes; GFP: Green fluorescent protein; HBV: Hepatitis B virus; IFN: Interferon; IV: Intravenous; IPSCs: Induced pluripotent stem cells; KLF4: Knppel-like factor 4; Lin28: Abnormal cell lineage 28; miRNA or miR: microRNA; MSCs: Mesenchymal stem cells; NK: Natural killer; NKG2D: Natural killer group 2 member D; NR: Not reported; Oct4: Octamer binding transcription factor 4; PEI: Polyethyleneimine; PSCA: Prostate stem cell antigen; Ref.: Reference; RFP: Red fluorescent protein; RVG: Rabies virus glycoprotein peptide; shRNA: Short hairpin RNA; shANGPLT4: Short hairpin angiopoietin-like protein 4; Sox: Sex-determining region Y-box transcription factor; sTNFR2-Fc: Soluble tumor necrosis factor receptor 2; TGFβ3: Transforming growth factor beta 3; TIPE2: Tumor necrosis factor alpha induced protein 8 family 2; TNF: Tumor necrosis factor; WDR5: WD repeat domain 5.