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REVIEW

Progress and Prospects: The design and production of plasmid vectors

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Plasmid DNA (pDNA) expression vectors are fundamental to all forms of non-viral gene transfer. In this review, we discuss principles of pDNA design and production including the impact of bacterially derived sequences on transgene expression and minicircle approaches to minimize their effects. The impact of inclusion of DNA elements such as scaffold matrix attachment regions (S/MARs), transcription factor (TF)-binding sites and tissue-specific promoters are described. The benefits of eliminating CG dinucleotides (CpGs) from the pDNA are also considered.
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In brief

Progress

- Large scale manufacture of pure, stable plasmid is required for gene transfer studies.
- Minicircles in which the backbone has been removed may offer advantages over conventional plasmids.
- Sequences in the bacterial backbone can be responsible for short duration of expression.
- Inclusion of a scaffold matrix attachment region (S/MAR) can overcome poor duration of expression in some systems.
- Incorporation of transcription factor-binding sites might aid plasmid translocation to the nucleus.
- Promoter selection is crucial to the level and persistence of transgene expression in different tissues.
- Plasmids free of CpG dinucleotides minimize inflammation and support sustained transgene expression.
- Integrating plasmids may offer advantages for stem cell targeting.
- Plasmids can be used to efficiently deliver and express shRNA molecules.

Prospects

- Plasmid manufacturing will be made more efficient and cost-effective.
- Industrial-scale minicircle production will facilitate further *in vivo* and clinical studies.
- New niches will be found for novel plasmid-based gene transfer systems.
- The mechanism(s) of transgene-silencing will be further clarified.
- Novel promoter/enhancer combinations will improve tissue specificity and safety.
- CpG-free technology will continue to be applied to plasmid-based vectors to minimize host inflammation.
- Integrating plasmid vectors such as Sleeping Beauty will be tested in the clinic.
- Sequences to promote intracellular trafficking will become increasingly important in plasmid design.

Introduction

Non-viral (plasmid-based) gene delivery can be used for gene therapy, DNA vaccination and the production of recombinant proteins in mammalian cells. The pDNA must be produced in a stable and relatively pure form, and can be delivered to cells either naked (carrier-free) by direct injection, electroporation, ultrasound and so on, or complexed with cationic lipids, polymers or peptides.[1]

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Until recently, research efforts have concentrated on modifying the carrier or delivery vehicle. However, non-viral efficiency can also be increased by improving gene expression from the plasmid (pDNA) component. Once inside the cell, the pDNA may need to escape subcellular compartments, such as endosomes, and at some stage must be released from its delivery vehicle. In a naked form, the pDNA is vulnerable to a potentially hostile cytosol before translocation to the nucleus where specific regulatory sequences facilitate gene expression. Thus, a full understanding of the intracellular pathway of pDNA can be helpful in designing strategies to overcome any rate-limiting steps.

Non-viral pDNA-based vectors have an almost unrestricted capacity for transgene DNA, and can accommodate

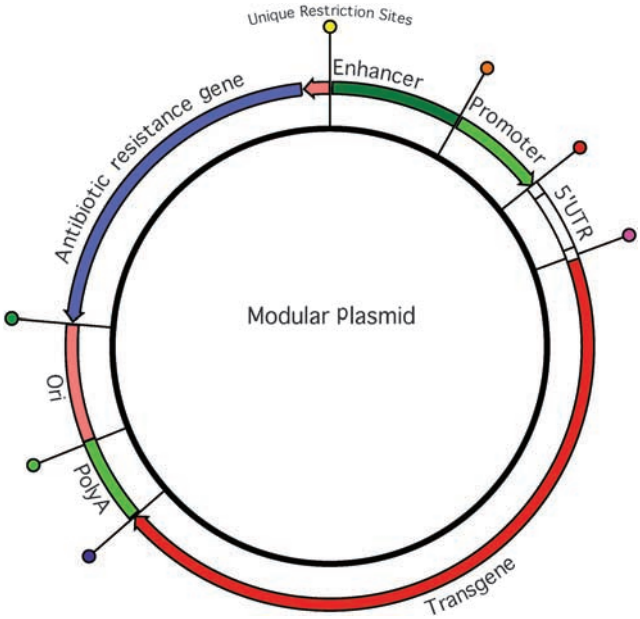

Figure 1 Diagram of modular plasmid design. Modular regions in a conventional plasmid can be considered in two halves. The mammalian expression cassette (EC) contains (clockwise from top): the mammalian or viral enhancer/promoter sequences for gene expression; 5′ untranslated region (5′UTR) including introns, reporter transgene or gene of interest and polyadenylation (polyA) sequence. The bacterial backbone (BB) contains a bacterial origin of replication (ori) and an antibiotic resistance gene or other selectable marker for plasmid amplification in bacteria. Ideally, modular regions would be separated by a unique restriction site for ease of manipulation.

large segments of genomic DNA for physiological regulation of expression where required.[2] Plasmid construction is relatively straightforward and permits the manipulation of a variety of regulatory elements that impact on gene transfer. For simplicity, an expression plasmid can be considered in two parts (Figure 1). The expression cassette (EC) is a transcriptional unit consisting of the gene(s) of interest and any regulatory sequences required for expression in mammalian cells. The bacterial backbone (BB) usually contains an antibiotic resistance gene and an origin of replication required for the production of the pDNA in bacteria.[3]

A major concern for the use of non-viral (pDNA-based) vectors is the short duration of gene expression observed in many animal models. If unable to replicate, or integrate into the cell genome, pDNA will be lost from daughter cells during successive divisions. However, even in slowly dividing or terminally differentiated cell types, where pDNA can remain as an episome (extrachromosomal) for the lifetime of the cell, transgene expression can still become silenced. Importantly, the mechanism of transgene silencing, which is incompletely understood, appears to vary depending on the choice of delivery vehicle and the target cell type(s).

Large scale manufacture of pure, stable plasmid is required for gene transfer studies

For *in vivo* gene transfer and vaccination studies, high-quality pDNA is required which should be predominantly supercoiled in form, relatively free from contaminating factors, such as bacterial DNA, RNA and protein, and stable with physical characteristics that remain relatively unchanged for long periods of time.[4] Conventional pDNA manufacturing processes are efficient for pDNAs up to 15 kb in size, but new technologies are required for large scale manufacture of larger plasmids, such as those containing genomic loci.[2]

It is well known that intravenously administered naked or complexed pDNA quickly decays from supercoiled to open circular forms, and that exposure to cytosolic nucleases can degrade pDNA. Increased susceptibility to degradation can be due to secondary structures within the pDNA, particularly those resulting in single-stranded regions susceptible to exo/endonuclease attack. Major hot spots were identified within the bovine growth hormone (BGH) polyA signal and within the ColEI *ori*, such that when polyA was replaced with synthetic or SV40 polyA sequences, the half-life of supercoiled pDNA was extended in cell lysates and mouse plasma.[5] These nuclease-resistant plasmids also showed improved stability of supercoiled forms stored in water over a 5-month period at 4 °C.[5] It remains to be seen if plasmids redesigned in this way result in improved transgene expression *in vivo*, especially as the selection of the polyA signal is also crucial for mRNA maturation/stability.[6]

Even in a nuclease-free environment, non-enzymatic, chemical strand breakage remains a threat to plasmid integrity. In an accelerated pDNA stability study, Ribeiro *et al*.[7] showed that pDNA secondary structure had the potential to both increase or decrease pDNA stability depending on the degree of under or overwinding of supercoiled DNA. Although it is generally believed that supercoiled or covalently closed circular plasmid is the physiologically active form, evidence for this *in vivo* is sparse. Recently updated US Food and Drug Administration and European Pharmacopoeia guidance on pDNA manufacturing for clinical use[4,8] provides detailed recommendations on pDNA purification, as well as strategies for cell banking and stability testing. However, our observations suggest that despite being prepared to an identical specification in line with regulatory guidance, pDNA supplied by subtly different manufacturing processes may vary to the extent that it affects efficiency *in vivo* (Figure 2), emphasizing that additional, uncontrolled factors probably contribute to performance.

Minicircles in which the backbone is removed may offer advantages over conventional plasmids

The deletion of unnecessary pDNA sequences is recommended to keep the pDNA molecule small and easy to manipulate. This also leads to increased numbers of plasmid molecules produced for a given mass of plasmid, thereby increasing the efficiency of production overall. Sequences in the BB that are required for production in bacteria, but not for gene expression in mammalian cells, could cause problems in patients, including the unregulated dissemination of antibiotic resistance genes and activation of cryptic expression signals.[8] One approach is to generate minicircles that lack the BB component, by including site-specific recognition

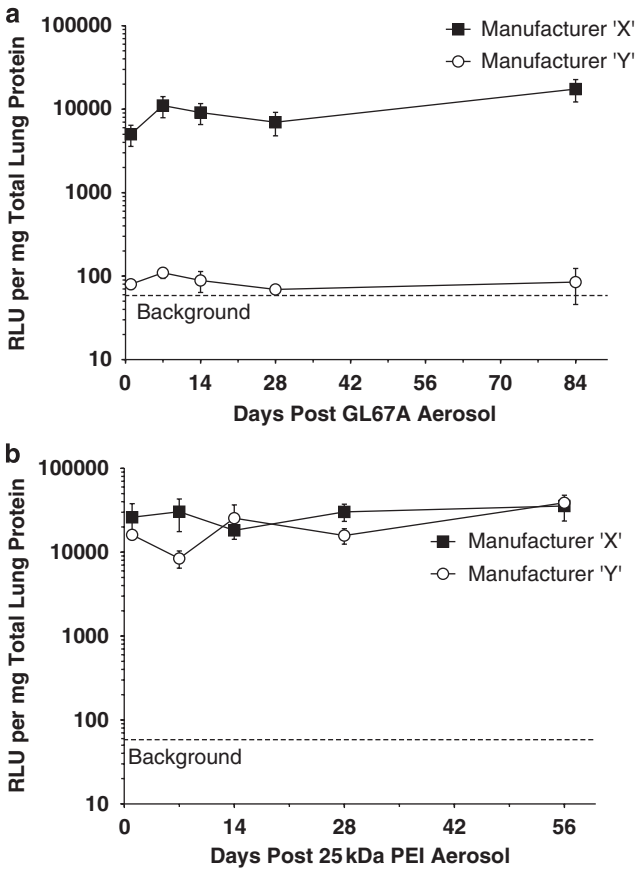

Figure 2 Comparison of *in vivo* performance using plasmid supplied by different manufacturers. Luciferase-expressing pDNA (pG1-hCEFI-soLux[15]) was supplied by two different manufacturers (X and Y) to the same specification and complexed with (**a**) GL67A cationic liposomes (26 mg pDNA; 10 ml), or (**b**) polyethylenimine (PEI; 2 mg pDNA; 10 ml) before aerosol delivery to the mouse lung (BALB/c; $n=6$ per group). Luciferase activity (RLU mg^{-1} lung protein) was similar for the two suppliers in the case of PEI, but varied by several logs when used with GL67A. The dashed line indicates background levels of luciferase.

sequences that can be recombined to generate two smaller supercoiled minicircles, one containing the EC, which can then be purified away from the other circle containing the unwanted BB sequences. Unfortunately, minicircle DNA will still contain unwanted sequences generated as a result of the recombination process.

Minicircles directing the expression of manganese superoxide dismutase (MnSOD) were evaluated for systemic radioprotection following intraoral or intravenous delivery to irradiated mice.[9] Mice receiving either plasmid or minicircles expressing MnSOD were similarly radio resistant, resulting in increased survival compared with controls. In addition, minicircles expressed sustained, high levels of interferon (IFN)-γ, sufficient for an antiproliferative effect in cells and tumours that was more efficient than the conventional parent plasmid.[10] These studies confirm the effectiveness of the minicircle approach *in vivo*. Although production of minicircle DNA is non-trivial and can be hampered by low recombination and purification efficiencies, a recent report described a highly efficient recombination process and novel affinity-based chromatography purification technology, based on the interaction of immobilized lactose repressor with multiple *lac* operator sequences on the minicircle.[11] This system may now offer the opportunity to scale-up efficient manufacture of large quantities of minicircle pDNA sufficient for clinical application.

Sequences in the BB can be responsible for short duration of expression

In vivo studies have shown that short-lived transgene expression is observed in the liver and lung even when pDNA is readily detected in transfected cells suggesting that the destruction of transfected cells is not responsible for the lack of sustained expression. The transcriptional silencing of gene expression signals, in particular the promoter, appears to be involved, although the precise trigger is unknown and may depend on the delivery vehicle and target tissue. High-pressure tail vein delivery of naked pDNA to the liver can result in transgene expression lasting only a few weeks, but is improved if the plasmid's BB is removed. Chen *et al*.[12] used a minicircle system to compare the effect of supplying the BB *in cis* (covalently linked to the EC) or *in trans* (separate), and showed that the covalent linkage of the BB sequences to the EC was essential for transgene silencing to occur; insulator elements were partially able to protect the transgene from being silenced. These data are consistent with the hypothesis that sequences in the BB can act as a focus for heterochromatin-associated histone modifications, which then spreads to adjacent EC sequences leading to transcriptional silencing.[13]

A similar mechanism was proposed for transgene silencing in herpes simplex virus-based vectors; minicircles devoid of prokaryotic sequences generated transgene expression in human fibroblasts and nude mice that was higher and more sustained compared with the parent pDNA.[14] Using Chromatin Immunoprecipitation (PCR analyses, bacterial sequences in the vector were shown to be associated with an inactive form of chromatin. However, even in the absence of the BB, expression from the HSV minicircle declined to background levels within 1 month suggesting that other factors could be involved.

The specific bacterial sequences responsible for this effect have not yet been identified, but unmethylated bacterial CG dinucleotides (CpGs) could play a role, as the reduction or elimination of CpGs from pDNAs can lead to improvements in the level and persistence of transgene expression in the lung.[15] CpG methylation of the pDNA before delivery has been considered, but this can lead to promoter attenuation, presumably through mechanisms similar to those observed after methylation of the mammalian genome.[16] Recognition of unmethylated CpGs present in the BB could trigger an innate immune response following detection in the endosome by toll-like receptor 9 (TLR9),[17] or by being targeted for methylation *in vivo*.[18] Rather than involving a specific sequence, transgene silencing could be triggered by the nature of chromatin, such as the lack of open chromatin configuration for active recruitment of transcription factors. Thus, the BB itself, which is not expected to be actively transcribed in mammalian cells, might trigger chromatin modification in this way. This hypothesis is also consistent with the histone modifications observed,

when the ubiquitously acting chromatin opening element, permits stable gene expression.[19]

Inclusion of a scaffold matrix attachment region (S/MAR) can overcome poor duration of expression in some systems

One strategy designed to overcome transcriptional silencing is the inclusion of an S/MAR, AT-rich sequences found in higher eukaryotic DNA, usually between genes that are thought to attach chromosomal DNA to the nuclear scaffold/matrix. Given the AT-rich content, S/MARs can serve as boundaries between active and inactive chromatins by ensuring that DNA remains lightly wound.[20] In addition to prolonging gene expression, S/MARs can, under certain circumstances, drive the replication of plasmids in a continually episomal state and have even been used to create transgenic animals.[21] A study to test pDNA containing an S/MAR sequence in the mouse liver following hydrodynamic injection[18] showed that the S/MAR was required for long-term gene expression when the liver specific α-1 antitrypsin promoter was used, but there was little evidence that the plasmid was actively replicating *in vivo*. Interestingly, analysis of DNA from mice that received a control plasmid (without an S/MAR) indicated that the α-1 antitrypsin promoter had been subject to CpG methylation in these mice, but S/MAR-containing plasmids remained free from methylation. These results show that pDNA can undergo *de novo* methylation in association with transcriptional silencing in an *in vivo* model and provide a method for insulation against such effects. However, robust and persistent pDNA transgene expression in the liver[12] and in the lung[15] has been reported in the absence of S/MAR elements.

Incorporation of transcription factor (TF)-binding sites might aid pDNA translocation to the nucleus

The design of many conventional pDNAs does not take advantage of additional DNA elements that could improve gene transfer. One example is the inclusion of sequences that bind to a TF to facilitate intracellular trafficking and/or nuclear import. A region of the smooth muscle γ-actin (SMGA) promoter that contains a number of smooth muscle-specific TF-binding sites has been shown to improve the uptake of pDNA following microinjection into smooth muscle cells in culture.[22] Mutation of specific TFs within the SMGA sequence decreased green fluorescent protein (GFP) fluorescence close to background levels and treatment with siRNA specific for the TFs also reduced nuclear import of pDNA.[22] This DNA sequence also dramatically increases gene expression *in vivo*.[23] When vectors containing the smooth muscle γ-actin sequence were delivered by electroporation into rat smooth muscle vasculature, GFP expression was observed at high levels after 2 days, but plasmids without the SMGA sequence had virtually undetectable levels.

Another example is the inclusion of nuclear factor (NF)-κB binding sites in pDNA[24,25] where the increase in NF-κB activation following gene delivery or activation with lipopolysaccharide could be used to increase trafficking of pDNA to the nucleus. Luciferase gene expression in the mouse lung vasculature was increased fivefold when NF-κB binding sites were included in the pDNA, but only when the pDNA was delivered complexed with a cationic liposome; when naked pDNA was used, no difference in gene expression could be observed.[24] This indicated that to use NF-κB to traffic pDNA to the nucleus, its activity had to first be elevated following the delivery of the cationic lipid carrier.

Although the concept of including so-called 'nuclear localization signals' remains compelling, many pDNA vectors lacking such signals still generate high and sustained levels of expression, indicating that there may be redundancy, and/or unidentified trafficking or nuclear localizing signals already present in commonly used pDNAs. Furthermore, although nuclear translocation of pDNA is considered rate limiting for transgene expression, a study involving PEI-mediated transfection of HEK293–EBNA1 cells revealed that despite the presence of pDNA in the nucleus of the majority of cells, only a subset of these cells were capable of transgene expression.[26] One might speculate that although these studies were performed in cell culture, they may also be relevant to the *in vivo* situation if slowly dividing cells experience periods of metabolic inactivity during which they might not be competent to express transgenes.

Promoter selection is crucial to the level and persistence of transgene expression in different tissues

The preference for viral promoters, capable of high-level but often short-lived transgene expression, has recently shifted towards selecting constitutively expressing, or tissue-specific endogenous promoters. Use of the human polyubiquitin C (UbC) promoter has resulted in sustained expression in the mouse lung, following the delivery of naked pDNA through electroporation,[27] and aerosol delivery of lipid complexes.[15] Synthetic promoter/enhancer combinations can also be successful in avoiding transcriptional silencing; replacement of the murine cytomegalovirus enhancer with the human version alongside a CpG-free elongation factor 1a promoter was successful in the mouse lung.[15] Similarly, in the liver, evaluation of reporter activity from four promoters following hydrodynamic delivery showed that the chicken β-actin/cytomegalovirus enhancer combination was a promising candidate.[28]

Tissue-specific promoters, which have been widely used in the viral gene transfer field, may offer improved specificity and safety for non-viral vectors. For example, the use of a hybrid promoter derived from the regulatory regions of the human achaete-scute homologue 1 and zeste homologue 2 genes restricted expression to non-small cell lung tumours,[29] and pituitary tumour-specific expression was successful with the rat growth hormone promoter.[30] Liver-specific expression following non-viral gene transfer was recently achieved with an α-fetoprotein enhancer/albumin promoter at levels that eclipsed the apolipoprotein enhancer/α-1 antitrypsin promoter element commonly used by viral vectors targeting the

liver.[6] Interestingly, the sustainability of liver-specific expression from this enhancer/promoter was markedly affected by selection of splicing elements positioned 3′ to the transgene,[6] although the value of splicing elements positioned 5′ to the transgene in non-viral ECs has been known for many years. Clearly, there is much to explore in using promoters that are responsive to specific diseases, such as local inflammation or tumour cells. A systematic evaluation of promoters is now required in animal models relevant to their intended application.

Plasmids free of CpG dinucleotides minimize inflammation and support sustained transgene expression

Although the absence of viral proteins in non-viral vectors can avoid triggering an immune response, the presence of unmethylated CpG dinucleotides in the pDNA can be recognized by the innate immune system through TLR9. Importantly, the DNA-sensing TLRs do not rely on structural differences to distinguish between pathogenic and host DNA, but instead depend on the presence of DNA in the TLR-containing endosome.[31] Thus, any exogenously delivered pDNA, which ends up in the endosomal compartment, for example, by lipid-mediated gene delivery, can be readily detected by TLR9, thereby stimulating an inflammatory cytokine cascade.

The removal of the BB from the vector using the minicircle approach can reduce the numbers of CpGs, but as unmethylated CpGs present in the EC are also stimulatory,[31] this is unlikely to eliminate the inflammatory response. To reduce the inflammatory consequences of lipid-mediated delivery of pDNA-containing unmethylated CpGs to the human lung,[32] pDNA vectors that are completely devoid of CpGs, not only in the BB but also in the entire EC, have been investigated.[15] Figure 3 compares the induction of inflammatory cytokines in a mouse lung model following lipid-mediated delivery of pDNAs with and without CpGs. CpG-free vectors not only dramatically reduced host inflammation but also supported sustained transgene expression in the mouse lung.[15] With the reducing cost of custom gene synthesis and the putative benefits of using CpG-free vectors *in vivo*, CpG-free technology can now be applied to a range of plasmid-based systems in other tissues.

Integrating vectors may offer advantages for stem cell targeting

Where the target cell population is actively dividing, or if stem cell targeting is required, the use of an integrating plasmid has advantages.[33] Both the phiC31 and Sleeping Beauty (SB) integrating systems have been extensively developed, and this year, early approval was given for the first gene therapy clinical trial using the SB system. An SB transposon carrying a CD19-specific chimeric antigen receptor will be transposed by electroporation into T cells from patients with CD19+-specific B-lymphoid malignancies.[34] Although efficiency of non-viral gene transfer is low compared with viral-mediated transduction, clinical grade pDNA can be produced quickly and for a fraction of the cost of retroviral vectors that have previously been used for such trials. Any integrating vector carries a theoretical risk of insertional mutagenesis and standard SB vectors exhibit random integration; one way to reduce this risk is to target integration at 'safe sites' in the genome. Studies are ongoing to create more targeted SB transposition by coupling a site-specific DNA-binding domain to the SB transposase.[35] However, the efficiency of these systems is

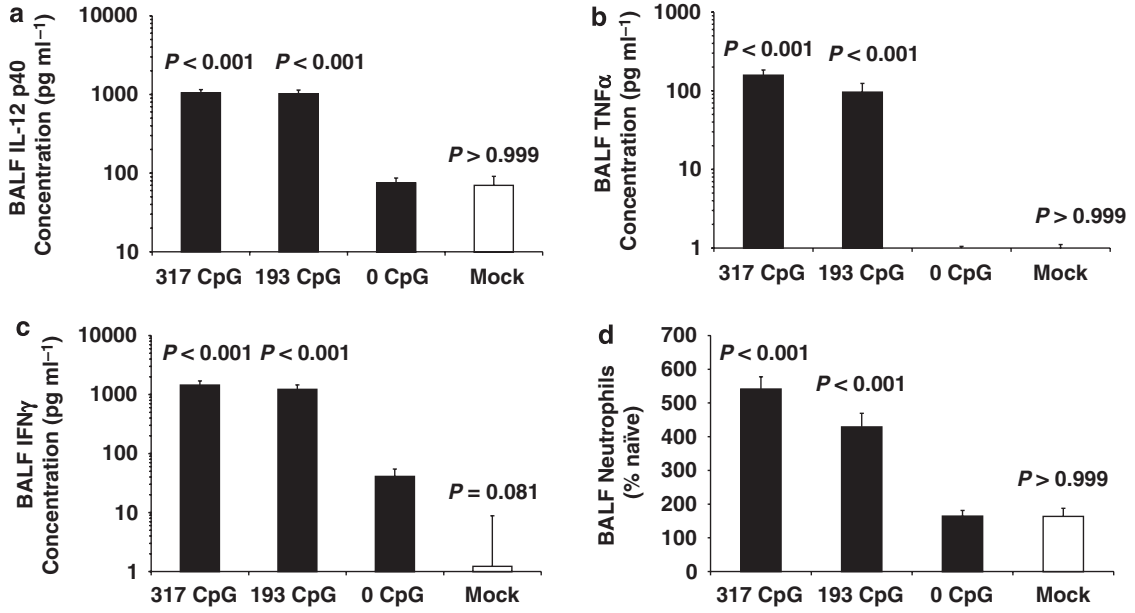

Figure 3 Inflammatory consequences of intranasal pDNA/cationic liposome delivery. Delivery of 80 μg of CpG-rich (317 CpGs/pDNA), CpG-depleted (193 CpGs/pDNA) and CpG-free (0 CpG/pDNA) pDNA complexed with GL67A cationic liposomes to the lungs by nasal instillation compared with mock vehicle delivery, as assessed by interleukin (IL)-12p40 (**a**), tumour necrosis factor (TNF)-α (**b**), interferon (IFN)-γ bronchoalveolar lavage fluid (BALF) levels (**c**) and BALF neutrophil numbers (**d**) 24 h after administration (BALB/c mice, mean ± s.e.m., $n = 5$–14 per group). Statistical differences shown are Bonferroni corrected Mann–Whitney *U*-tests compared with the unlabelled comparator after significant Kruskal–Wallis analyses.

currently low and they have yet to be rigorously tested in relevant *in vivo* models. Similar to retroviruses, SB vectors can be prone to post-transcriptional gene silencing, likely due to factors including the choice of promoter, transgene sequence and the specific integration site.[36] However, silencing in this system has been shown to be associated with *de novo* methylation of CpGs within the transposed element;[37] thus the removal of CpGs could improve the duration of expression from SB vectors.

Plasmids can be used to efficiently deliver and express short hairpin RNA (shRNA) molecules

Optimizing pDNA design for expression can benefit other applied technologies, such as pDNA vaccination strategies[38] and transient transfection of mammalian cells, for the production of recombinant proteins.[26] Plasmid-based vectors can also be used to deliver shRNA molecules to induce specific gene silencing with the potential to treat aberrant gene disorders and viral infections.[39,40] Plasmid elements that affect the expression and potency of shRNA molecules include selection of enhancer/promoter sequences, shRNA hairpin stem length, shRNA loop design, control of shRNA transcript 3′ end, inclusion or otherwise of a structural RNA scaffold and the incorporation of linker/recombination sequences to facilitate vector construction.[41] Polymerase III (PolIII) promoters, such as U6 and H1, that have well-defined transcription initiation and termination sites facilitate precise shRNA design and remain the standard for use in driving shRNA expression. However, the high and constitutive transcriptional activity of polIII promoters can lead to the saturation of the endogenous miRNA processing factors, such as RNA induced silencing complex (RISC) and exportin-5, that are required for efficient shRNA processing, which in turn can lead to cytotoxicity and tissue damage.[42] Although additional regulatory elements can be combined with polIII promoter elements to restrict their transcriptional abundance,[43] such approaches typically rely on the coexpression of additional protein elements that may well be recognized as foreign by the ultimate host.

Consequently, the use of polymerase II (polII) promoters for shRNA expression is receiving renewed interest; greater control exists over the abundance of polII promoter-derived transcription, and several polII enhancer/promoter elements have well-defined tissue-specificity, both clearly attractive features. The general use of polII promoters for shRNA expression has been limited by the poorly understood requirements of the flanking regions necessary for efficient transcription and 3′-end processing. However, careful optimization of the distance from the promoter element to the shRNA hairpin, and from the hairpin to the transcriptional termination site, has facilitated the construction of highly efficient polII-based shRNA pDNA expression vectors.[44] In particular, the use of 3′ RNA processing elements from snRNAs or tRNAs, rather than synthetic polyadenylation sequences, permits greater control over final shRNA structure, leading to the enhanced knockdown efficiency.[44,45]

Whereas early studies revealed that shRNA hairpin lengths of up to 29 nucleotides offered enhanced knockdown efficiency, more recent studies have highlighted the importance of shRNA loop length rather than hairpin length.[46] As an alternative, many have turned to embedding shRNA sequences within a miRNA scaffold. As endogenous miRNAs are often transcribed from polymerase II promoters, they are readily amenable to polymerase II-based pDNA expression systems. Several commercial pDNA libraries now exist that offer genome-wide RNA interference in vector systems that can readily be utilized as either pDNA expression systems, or converted to retroviral or adenoviral vectors.[47]

Prospects

The opportunity now presents itself to specifically focus on the rational design of pDNAs to enhance the safety and gene expression profile for many non-viral vectors. Novel endogenous and synthetic promoter sequences will continue to be investigated to fine tune gene expression for the target tissue, although the synergistic effects of adjacent sequences including enhancers, terminators and the BB will need to be evaluated. The development of cost-effective large scale production of minicircles should encourage their use in the clinic and in animal models to alleviate transcriptional silencing and promote persistent transgene expression. Importantly, clinical trials will establish if the CpG depletion of pDNAs leads to fewer side effects in human subjects and whether CpG-free plasmids should be recommended for all clinical pDNAs.

Note added in proof: Please note that corrections have been made to this article since initial online publication on 8 January 2009. Due to a typesetting error, the axes of Figure 2 were labelled incorrectly in the original version; however, the labelling has now been amended and this is the corrected version of the article, incorporating the revised figure. The Publishers would like to apologise for any inconvenience caused.

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