CELL & GENE THERAPY INSIGHTS

NEXT GENERATION VECTORS

SPOTLIGHT

EXPERT INSIGHT

# *Minicircles*: next-generation gene vectors

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Plasmid DNA is commonly used in vaccination, cell and gene therapy, and as a basic substance in viral vector and RNA production. Backbone sequences in a plasmid vector are only needed for amplification in bacterial cultures. Since the uncontrolled expression of these sequences may have profound detrimental effects, for example, the dissemination of antibiotic resistance genes, an important goal in vector development is to produce supercoiled DNA lacking such bacterial backbone sequences. One elegant approach is minicircle (MC) DNA, consisting almost only of (therapeutically) active gene cassette. Over the past few years, MCs have proven to be a reliable tool for efficient transgene expression in eukaryotic cells both *in vitro* and *in vivo* as well as for *ex vivo* modification for cell therapy or lately even for the generation of induced pluripotent stem cells. Recent trends and progress in pre-clinical studies suggest that the time has come for preparation of such minimalistic vectors to a High Quality Grade to enable for example the production of viral vectors for gene therapy. Furthermore, significant developments in transfection efficiency of non-viral vectors suggest that GMP grade MCs conformant to regulatory guidelines would be needed in the near future for direct clinical applications. This article provides an overview of the advantages and drawbacks of different approaches to produce MC DNA, their applications, and finally describe current and future developments.

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Since the European Medicines Agency (EMA) proposed in their guidelines for medical gene transfer products the avoidance of selection markers such as resistance against antibiotics [1], minicircles (MCs) have come to the fore as a promising tool for the production of future pharmaceuticals used in gene therapy and vaccination.

MCs derive from parental plasmids (PP) with antibiotic resistance markers, the gene of interest (GOI) and origin of replication (ori), as well as two special signal sequences

right and left of the GOI. An intra-molecular recombination process results in the supercoiled covalently closed circular (ccc) GOI (Figure 1).

After successful recombination, the MC has to be isolated from a mixture of three types of circular DNA molecules: minicircles, miniplasmids and possibly residual amounts of parental plasmids.

## PRODUCTION & PURIFICATION

MCs are defined as circular, supercoiled non-viral DNA molecules derived from a plasmid DNA but free of bacterial sequence elements, which are irrelevant and/or detrimental for gene therapy applications or DNA vaccination [2]. A PP is generated that contains the eukaryotic gene of interest under the control of a promoter sequence and preceding a polyA signal. This target gene, gene cassette or just the sequence of interest is flanked by recognition sites for *in vivo* recombination. MC production is a two-step process: firstly, an *in vivo* recombination that results in the conversion of the supercoiled PP into a supercoiled replicative miniplasmid (MP) and a non-replicative supercoiled MC (Figure 2). The MP contains the prokaryotic sequence elements (e.g., selection marker, ori) whereas the MC is free of any bacterial sequence except for a short residual sequence, namely the sequence for chromatography, affinity and recombination (SCAR) [3,4] – consisting of identification sequences and one recombination sequence resulting from the cis-recombination of the PP (Figure 1). The supercoiled topology is critical for transgene expression in mammalian target cells [5,6]. Various approaches, all based on recombination (see below), were presented during the last few years, but only those resulting in an efficient recombination and that worked unidirectionally were able to generate a recombination product (RP). Unidirectional means that the recombinase reaction is driven in one direction rather than being able to recombine back and forth. This can be achieved by either choosing a recombinase that is working from a PP into two circles (MP and MC) by nature or, as was shown by Bigger *et al.* [7], by the manipulation of the recombination sites within the PP resulting in hybrid sequences on the MC and the MP that are unable to participate in further recombination events or at least have a reduced efficiency to do this. After this was solved for some recombinases it was important that the subsequent quantitative removal of the undesired MP could be realized to obtain pure MC. This was achieved via the second step, dealing with the chromatographic purification that aims to separate the two closely identical DNA molecules, so that the MC alone forms the final product (Figure 3) [8].

In the most successful approach presented thus far, the induction of *in vivo* recombination during the exponential growth phase was achieved by the addition of L-arabinose to drive the pBAD promoter [3]. This basic technology dealing with expensive affinity chromatography technology was modified by PlasmidFactory, resulting in a scalable process for the production of MC of significantly different sizes ranging from MC.shGFP of 501 bp [9] to


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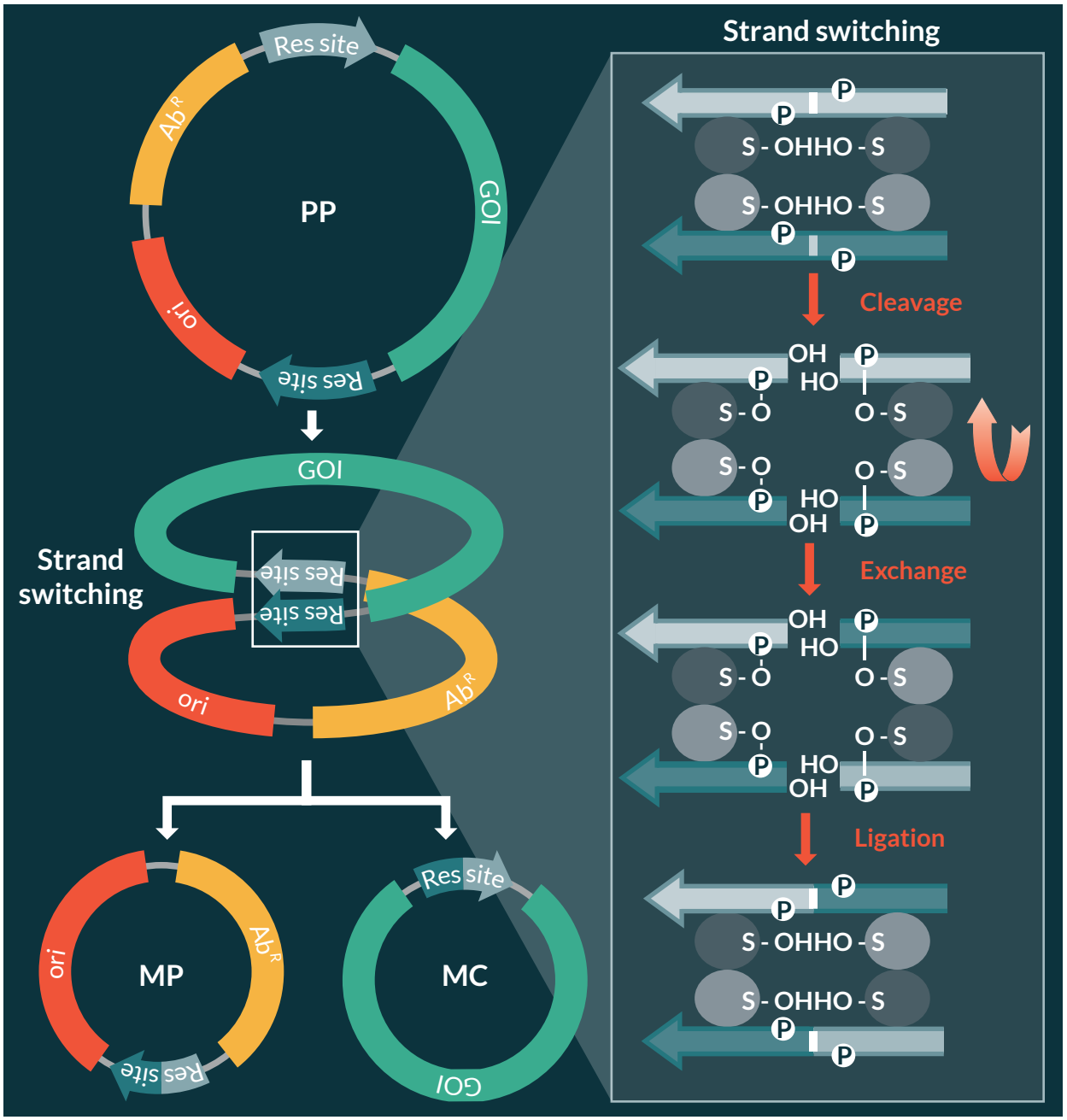
## FIGURE 1

**Schematic for *in vivo* recombination at 'Res' sites.**




Recombination of parental plasmid (PP) into miniplasmid (MP) and minicircle (MC). The Res sites are present in head-to-tail orientation. Inset shows a detailed view of the mechanism of strand switching as described by Stark [55]. Briefly, each Res site binds a recombinase dimer (shown as dark or light grey ovals). The active site serine residue of the corresponding recombinase subunit is responsible for creating a phosphodiester break in each of the four strands. A subunit rotation of one half of each dimer with respect to the other half about the axis as shown in the figure results in strand switching. The phosphodiester breaks are re-ligated and the recombination is complete. For more details on the molecular mechanism, the reader is referred to Stark [55].

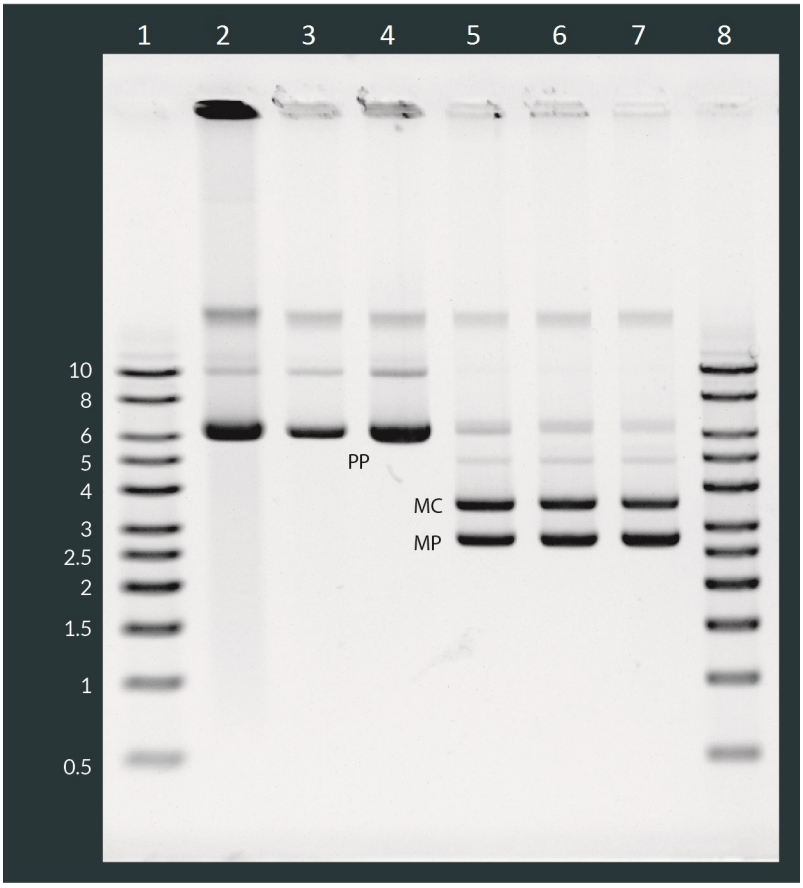
MC.DP2rs of 21870 bp [10] (Figure 4). This demonstrates that on the one hand an extremely small MC can be generated and on the other, even extremely large sequences are able to form a MC.

As a final step several analytical techniques are applied and the purification is checked in order to ensure product quality on critical parameters such as MC topology, homogeneity and levels of residual

chromosomal DNA and lipopolysaccharide contamination [8]. One important feature of the MC produced this way is that the molecule keeps most of its superhelicity. This means that the resulting MC is a ccc molecule, which might be the reason for a superior expression profile compared with plasmids or linear DNA molecules [8,11]. It further turned out recently that the technology applied here is resulting in an almost complete monomeric supercoil, while the other systems (ϕc31/att, λ-int, cre-lox) either create inhomogeneous back-and-forth recombination products or multimeric MCs with inconsistent quantities of the respective topologies.

**FIGURE 2A**

**Recombination of parental plasmid to miniplasmid and minicircle.**




Agarose gel electrophoresis of samples from *in vivo* recombination of parental plasmid. Lanes 1 and 8 show DNA standard marker from PlasmidFactory GmbH & Co. KG. with molecular sizes shown in kb. Lanes 2 and 3 – preculture; Lane 4 – main culture, pre-induction; Lanes 5, 6 and 7 show 30 min, 60 min and 120 min post-induction, respectively.

## DEVELOPMENT OF MINICIRCLES

Two enzyme families have been used so far to achieve the recombination process: tyrosine or serine recombinases as integrase, Cre recombinase, FLP recombinase and ParA resolvase. One of the earliest patents awarded directly for the synthesis of MCs through recombination of a parental plasmid is WO 96/26270 [12]. A modification by Bigger *et al.* in US 11/249929 described the use of mutated recombination half-sites that drove the recombination in a unidirectional manner resulting in better MC yields but a high amount of concatamerization was still observed [7]. The recombination events of the Cre recombinase (lox sites) and of the FLP recombinase (FRT sites) result in identical or highly similar sites and thus the recombination is bidirectional and fully reversible, finally resulting in several multimer structures due to intramolecular and intermolecular recombination [13,14].

The lambda integrase is known to favor unidirectional recombination between attB and attP sites [15]. The resulting molecules carry attL and attR sites. Here, still around 30% of the MCs are multimers.

The recombination driven by the integrase of bacteriophage PhiC31 is strictly unidirectional [7,16]. This enzyme mediates recombination events between an attP and an attB site, resulting in recombination products containing attL and attR sites. However, the recombination efficacy is still very low.

The ParA resolvase mediates only intramolecular recombination through its resolution sites [3]. Hence, the recombination is unidirectional to completion and no multimers occur.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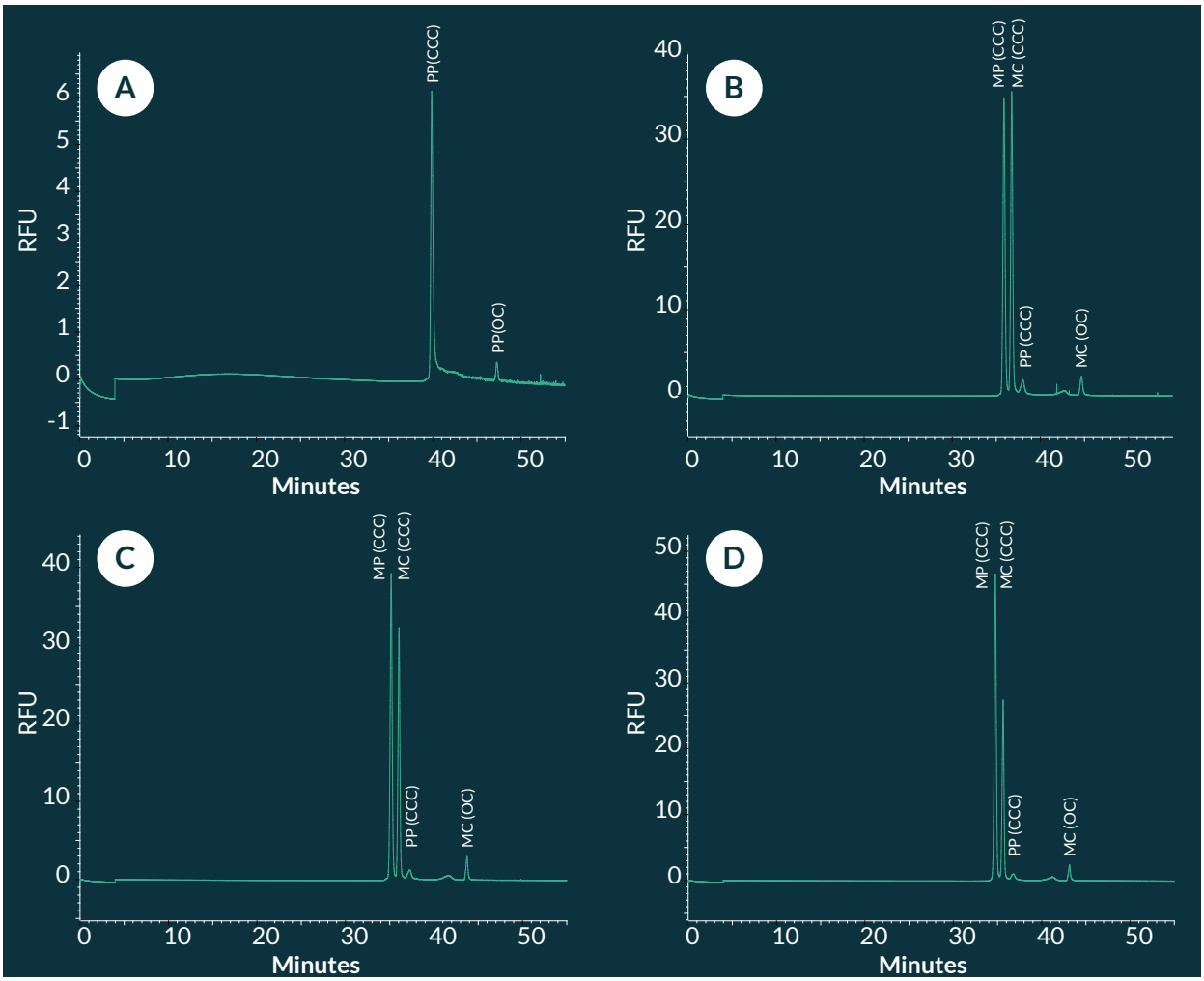
## FIGURE 2B

**Recombination of parental plasmid to miniplasmid and minicircle.**




Capillary gel electrophoresis (CGE) of samples from *in vivo* recombination of parental plasmid. (A) Supercoiled (CCC) and open circle (OC) conformations of parental plasmid in the preculture. **(B)** 30-minute post-induction, strong signals corresponding to the supercoiled miniplasmid and minicircle are detected. **(C)** 60-minute post-induction, the miniplasmid is relatively higher in amount than the minicircle due to the replicative ability of the former and the continuing cell division. **(D)** 120-minute post-induction, the reducing peak of MC shows that longer incubation times result in dilution of the minicircle among the cell population whereas the miniplasmid continues to replicate. The parental plasmid is reduced to a short residual peak. The scale in **(A)** is lower due to a lower concentration of the sample analyzed. This has no effect on the composition.
MC: Minicircle; MP: Miniplasmid; PP: Parental plasmid.

Developments in MCs are described by Bode [14,17], Mayrhofer [3], Kay [16,18–20] and a more detailed analysis of MC is provided by Grund and Schleef [21]. Table 1 shows the various recombination systems employed for the production of MCs. The different systems summarized here vary considerably in their efficiency of recombination and directionality.

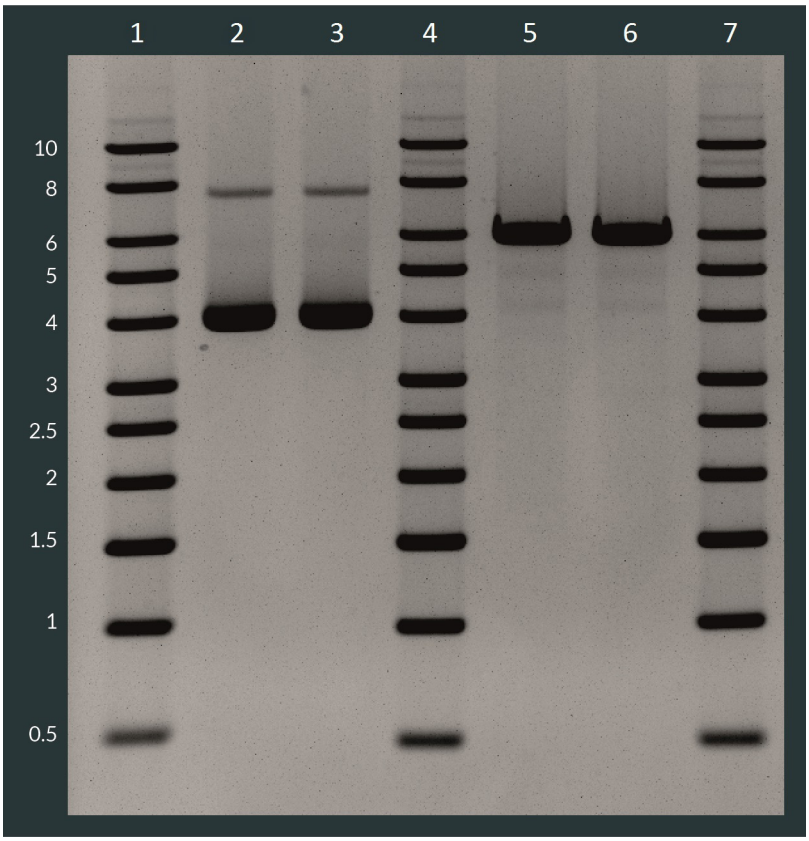
## REGULATORY STIPULATIONS DRIVE THE NEED FOR MC DEVELOPMENT

### Avoidance of backbone sequences

Antibiotic-resistance genes on plasmid DNA vectors, which function as selection markers during vector development in bacterial hosts, are a biosafety risk for application in gene therapy. Recombinant viral

**FIGURE 3A**

**Purified minicircle as final product.**




Agarose gel electrophoresis of purified minicircle from Figure 2 for Quality Control (QC) of final product. Lanes 1, 4 and 7 show DNA standard marker from PlasmidFactory GmbH & Co. KG. with molecular sizes shown in kb. Lanes 2 and 3 – purified minicircle as a final product. Lanes 5 and 6 – linearization of minicircle by restriction digestion with a single-cutter enzyme. Molecular size: 5775 bp. The electrophoresis for Quality Control (QC) was carried out in a longer gel that reduced the effective V/cm by 50%. This resulted in the MC band (ccc) appearing at a slightly higher position compared to Figure 2A.

vectors produced using prokaryotic tools may contain unintentionally packaged resistance gene sequences that may be carried over to target tissues and persist *in vivo* [22].

A significant risk factor is the expression of these genes in mammalian cells under the control of mammalian promoters leading to unpredictable and undesirable metabolic changes. For example, this was shown to manifest *in vitro* in fibroblasts as a change in the level of glycolytic metabolites and an increase in the mRNA level of an endogenous transcription factor c-myc, which has a potential to disrupt the regulation of various other genes [23]. It is therefore necessary to look for alternatives in order to prevent the prevalence of these resistance genes and cryptic expression by mammalian promoters, avoid the risk of horizontal gene transfer to human microflora and the risk of rising multidrug-resistant pathogens [24]. Strategies for avoiding prokaryotic sequences in DNA vectors for gene therapy by getting rid of the antibiotic-resistance genes and achieving selection through alternative antibiotic-free mechanisms have been reviewed by Schleef [25]. The MC technology takes this a step further and removes the prokaryotic origin of replication as well, resulting in a molecule that is almost free of any bacterial sequences.

Vertebrate genomes are generally CpG-deficient [26]. A common epigenetic feature is the high rate of methylation of 5′-cytosine in CpG. This methylated base, can over time spontaneously deaminate into thymine which cannot be efficiently corrected by DNA-repair enzymes. This has led to an underrepresentation of CpG dinucleotides in the mammalian genome (CpG islands occurring near transcriptionally active regions being an exception) [27]. Conventional plasmid DNA vectors used in non-viral gene therapy contain bacterial backbone sequences with CpG motifs that occur at the statistically expected frequency. These unmethylated CpG dinucleotides represent a risk factor for safe clinical application due to their immunogenicity [28]. Thus, the removal of bacterial backbone sequences and thereby any unmethylated CpG sites contained in them, results in the better safety profile of MCs.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## Efficiency of transgene expression

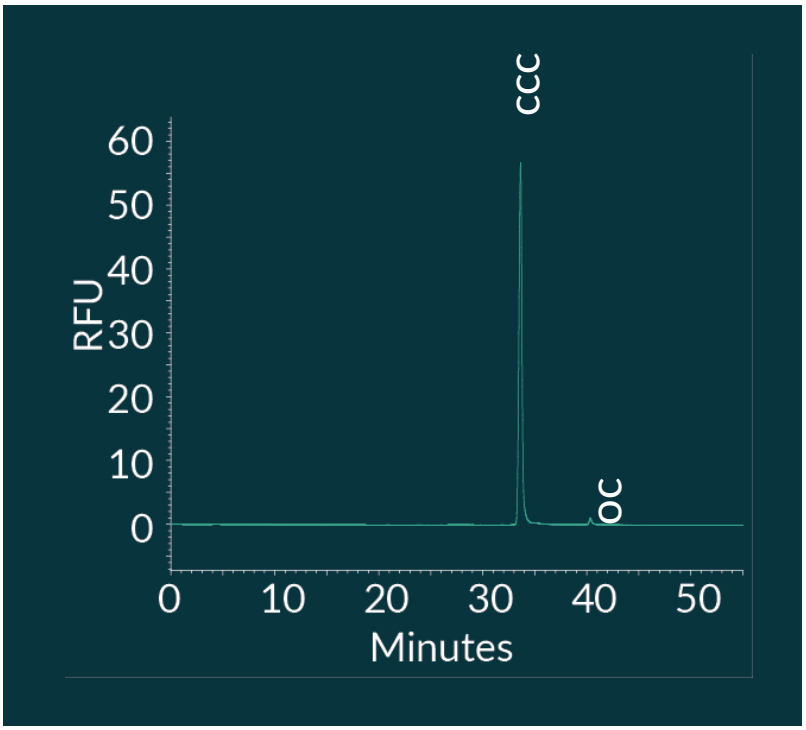
One major drawback of non-viral DNA vectors is the phenomenon of epigenetic gene silencing. It occurs after successful gene transfer has taken place and even when the presence of copies of the transgene are detected and confirmed. This results in initial high levels of transgene expression, which drop rapidly thereafter, leading to transient unsustainable expression.

CpG methylation of the vector prior to delivery may result in promoter silencing in a mechanism similar to that in a typical mammalian cell. As a result, CpG-free plasmids were developed, which were capable of sustained transgene expression and a reduction in the inflammatory response through Toll-like Receptor 9 (TLR9) [29,30]. Nevertheless, Chen *et al.* argue that creation of a CpG-free plasmid would not completely mitigate the problem of silencing [18]. Furthermore, they present the hypothesis that epigenetic gene silencing is a result of chromosome structure in the mammalian cell, which is indirectly influenced by the type of DNA sequence. Plasmid backbone DNA lacks mammalian promoter sequences or transcription factor binding sequences and are therefore prone to conversion into transcriptionally inactive heterochromatin structure upon nuclear entry. This structure could spread out to the cis adjoining transgenic expression cassette leading to transgene silencing. On the other hand, MCs possess exclusively mammalian-active promoter regions driving the expression cassette and are prone to conversion into transcriptionally active euchromatin structures leading to stable transgene expression [18]. In these cases, a covalent connection of the bacterial plasmid backbone to the transgenic expression cassette was shown to be required for silencing and that the backbone does not silence the transgene in trans [18]. An interesting line of thought has been put forward by Lu *et al.*, wherein it was demonstrated that the silencing of DNA vectors *in vivo* has more to do with the length of the extragenic DNA sequence between the 5′ and 3′ ends of the transgene expression cassette and not directly the sequence itself or its origin. Thus, even a MC could be silenced *in vivo* to an extent as with plasmid DNA, by including 1 kb of DNA sequence that could even be random, as an extragenic backbone [19].

Comparison with plasmid DNA vectors showed that MCs coding for

**FIGURE 3B**

**Purified minicircle as final product.**




Capillary gel electrophoresis (CGE) of minicircle from Figure 3A for QC. The final product contains 98.7% of the minicircle in the supercoiled (CCC) conformation.

reporter genes could be transfected through diverse methods. They consistently showed more efficient transgene expression in human melanoma and colon carcinoma cell lines for luciferase (up to a maximum of four-fold) and GFP (up to 3.6-fold increase in GFP-positive transfected cells). *In vivo* injection of lacZ-containing plasmids or MCs into subcutaneous A375 tumors in nude mice showed that whereas the former caused intensive staining in small restricted pockets, the latter showed broad areas with variable staining intensities thus proving the efficiency of MCs to transfect large areas of tissues [8,11].

A smaller size of the DNA vector has been shown to result in better cellular and nuclear entry after electroporation [5]. Advantages of MC over plasmid due to their reduced size was tested in electroporation experiments to deliver the luciferase reporter gene into melanoma or fibroblast cells. For the fibroblasts under suboptimal electrotransfer conditions, equimolar amounts of minicircle fared better than the plasmid [31]. Different alterations to a plasmid DNA vector have been reported in order to improve stability, cellular uptake, nuclear transport and ultimately better transgene expression. A smaller size results in better transfection efficiency due to more efficient diffusion into and inside the cell and hence also a better efficiency in expression [11,31]. Reduced size of vector donor, as in the case of MC, was also shown to give better transposition activities for *Sleeping Beauty* (SB)-mediated transposition [32,33]. It has been shown through RT-qPCR that MCs mediate a significantly more effective transgene transcription than their corresponding plasmids [8].

## MCs FOR NON-VIRAL GENE THERAPY

MC vectors present themselves as safe and ideal non-viral vectors for both quiescent and dividing cells, in particular along with a better performance than standard non-viral vectors such as plasmids [34]. Following entry into the target nucleus, a plasmid vector is expected to remain episomal and result in transient expression. For transient expression of a therapeutic gene, an extended expression could in certain cases be beneficial. Efficient transfection of MCs into embryonic stem cell-derived neural stem cells through microporation was reported, and resulted in expression of transgene in 75% of the cells along with a higher cell viability in comparison to plasmid-based vectors. Additionally, the MCs were maintained in a higher copy number and were expressed for a longer duration [35]. In a recent *in vivo* study, MCs delivered by hydrodynamic injection were shown to be efficient in episomal maintenance and expression of a codon optimized *pah* gene in the liver of a mouse model of human phenylketonuria. MCs offered freedom with the size of the therapeutic transgene construct including an intron sequence and the native promoter-enhancer. Moreover, they required only a lower dosage and could be confirmed to remain episomal while yielding sustained PAH activity in the liver [36].

Their excellent safety profile notwithstanding, a major limitation with non-viral vectors is their poor gene transfer efficiency in comparison to viral vectors. The successful translation of the benefits of MCs for non-viral gene therapy will thus depend largely on the concurrent development of efficient gene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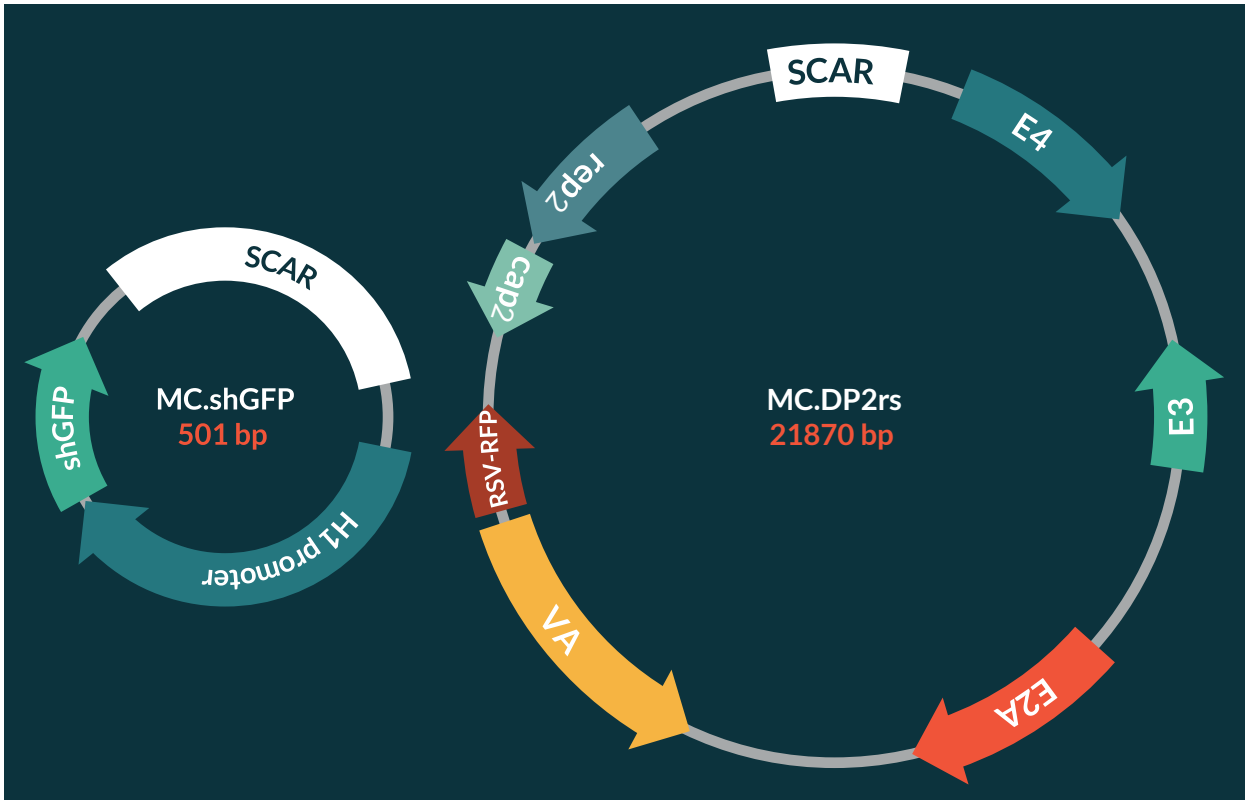
## FIGURE 4

**Minicircles of significantly different sizes ranging from MC.shGFP of 501 bp to MC.DP2rs of 21870 bp.**




Representation of the range in sizes of minicircles produced by PlasmidFactory GmbH & Co. KG, Germany. SCAR contains Res site and sequence for purification. MC.DP2rs is a minicircle of 21870 bp with helper and packaging functions for AAV serotype 2 and red fluorescent protein [10]; MC.shGFP is a minicircle of 501 bp carrying the sequence for an shRNA against GFP [9].

delivery methods. Various strategies currently under consideration, their advantages and drawbacks as well as potential for improvement have been summarized by Gaspar *et al.* [34]. These include, among others, chemical carriers such as synthetic polyionic polymers and physical methods such as electroporation. It is critical here to consider not only the delivery into the cells but also nuclear uptake of the MC DNA vector [5,34].

## S/MAR elements

Second-generation non-viral 'chromosome-based vectors' were developed in order to overcome the difficulties with viral vectors and also to address the problem of loss of transgene expression. Linear DNA is particularly susceptible to epigenetic silencing and this drove the search for stable episomal circular vectors [37]. For efficiently modifying dividing cells, maintenance problems have to be addressed too. Loss of recombinant vectors in mammalian cells due to plasmid dilution could be prevented by so-called 'mammalian ORIs' such as the EBV and SV40 [38]. However, they require the continuous supply of trans factors from the target cell such as EBV nuclear antigen 1 and the SV-40 large-T antigen respectively, which is not always easy and when supplied, could cause undesired consequences [37]. In this view, it is attractive to establish a MC through a Scaffold Matrix Attachment Region (S/MAR) element and also empower it with non-viral

**TABLE 1**

**Site-specific recombinases used for MC production.**

| Study title | Recombination elements | Enzyme | Family | Ref. |
|---|---|---|---|---|
| A new DNA vehicle for nonviral gene delivery: supercoiled minicircle | attP, attB | Bacteriophage λ-integrase | Tyrosine | [15] |
| An *araC*-controlled Bacterial *cre* Expression System to Produce DNA Minicircle Vectors for Nuclear and Mitochondrial Gene Therapy | loxP | Bacteriophage P1- integrase (Cre recombinase) | Tyrosine | [13] |
| Minicircle DNA Vectors Devoid of Bacterial DNA Result in Persistent and High-Level Transgene Expression *in Vivo*. | attP, attB | Bacteriophage ϕC31- integrase | Serine | [16] |
| Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells. | FRT | Yeast 2 µm circle- FLP recombinase | Tyrosine | [14] |
| Minicircle-DNA production by site specific recombination and protein–DNA interaction chromatography. | res | ParA resolvase from pRK2 or pRP4 | Serine | [3] |

replicative functions. Establishment could be seen as the process that ultimately results in the functional coupling of the episome together with the host nuclear substructures. The critical aspect here is that the S/MAR element is coupled to an adjacent transcription unit [39].

In this context, the use of S/MAR elements for episomal transgene expression in target cells could be further enhanced by the combination of the MC technology which was first demonstrated in cell lines using the FLP route by Nehlsen *et al.* [14]. A later report by Argyros *et al.* [40] showed persistent transgene expression in a mouse model. In this case, the Cre site-specific recombinase acting on the 34 bp direct repeat loxP sites flanking an expression cassette, was used to create two topologically distinct supercoiled DNA molecules out of a single precursor plasmid. Since the two original sites were chosen to be mutant, the resulting loxP sites were either hybrid (present on the MC) or wild-type (on the MP) and thus the Cre-recombination reaction could be shifted from equilibrium towards MC production and the problem of reverse recombination could be mitigated. This bolstered the field nonviral gene therapy by bringing a potential solution to the problem of transient expression caused by the phenomenon of gene silencing even upon maintenance of the DNA vector. Interestingly, in the absence of selection pressure *in vitro*, it was found that both the minicircle form of the vector as well as presence of the S/MAR element were essential for the episomal establishment and for sustained reporter gene expression. *In vivo*, liver-targeted MCs containing an S/MAR element showed a significant increase in transgene expression [17,39]. Additionally, multiple replicative minicircles could be established simultaneously in a host cell, thus enabling the simultaneous expression of multiple target genes [39].

## Embryonic gene transfer

Injection of S/MAR-containing MCs into the cytoplasm of bovine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embryos was shown to be a simple and efficient method for expressing marker genes as well as functional genes from episomal vectors thus improving the toolbox available for the development of genetically modified mammalian animal models [37]. Although creation of germline-modified mice was possible through delivery of SB MC of the Venus reporter gene (3.7 kb) and MC for SB100X transposase (2 kb) into cytoplasm of murine zygote, further optimization of the experimental conditions is required to improve the transgenesis frequency [41].

## CHALLENGES IN PRODUCTION & PURIFICATION

The *in vivo* site-specific recombination of PP into MP and MC is an efficient process, but nevertheless has much potential for improvement. The first and obvious desired step is the rapid multiplication of the parental plasmid during the growth of the culture. Strategies have been described that try to increase the PP yield and therefore the starting material for recombination, for example for plasmids with a pBR322 origin. Similarly, there is scope for optimizing the time-point and duration of induction for individual cases through close monitoring of the recombination process [42]. After induction, any residual unrecombined PP in the cells and the MP, which is a replicative side-product, cause difficulties during downstream purifications and would act as contaminants of the final product and decrease its quality. The major factor compounding the purification problem is the broad similarity in the properties of the three DNA molecules – PP, MP and MC – during chromatography.

The recombination reaction by ParA resolvase is marked by a high efficiency and is unidirectional [43]. Since the reaction proceeds to completion, there are no unrecombined parental plasmids left in the cells [3]. This facilitates the downstream purification steps, since only the MP has to be separated from the MC product. An idea for *in vivo* degradation of PP and MP described already by Bigger *et al.* [7], is implemented in the recombination method using the ϕC31 integrase, which combines the expression of an endonuclease with target sites on the miniplasmid region, thereby resulting in the degradation of both MP and PP even before the purification process [20]. In another approach, the lack of supercoiling was (at least) compensated by applying *in vitro* superhelicity to the MC molecule [44].

The purified MC product is also subjected to quality control (QC) procedures similar to those for a typical plasmid. One powerful technique to analyze plasmid topologies is the Capillary Gel Electrophoresis (CGE), which provides information on the homogeneity of a DNA preparation (Figures 2 & 3) [45,46]. Interdisciplinary collaboration has in recent times yielded significant technological advances for downstream purification of therapeutic DNA products. Drawing from the strengths of nanofluidics, a dielectrophoretic separation principle was demonstrated for the label-free separation of MCs from parental plasmid and MP, thus promising to be a QC tool in the production of DNA vaccines [47]. In another example, application of the principle of Surface Plasmon Resonance to screen for biomolecular interactions led to the finding that immobilized

arginine dipeptide ligands may have either a high or low affinity to minicircle DNA under specific buffer conditions [48].

## LATEST DEVELOPMENTS

A summary of pre-clinical studies involving MC vectors on various animal models dealing with a wide variety of disease targets is provided by Gaspar *et al.* [34].

Purity of engineered viral vectors for gene therapy is a particularly important parameter both in the context of quality control and safety of ATMP according to regulatory authorities [49]. The risk of AAV vectors carrying prokaryotic sequences capable of eliciting an inflammatory response in the target cells or transfer of antibiotic resistance genes to the human microbiome [22,29,50], drove the need to adapt the MC technology for AAV vector production. In a landmark study published last year, MC equivalents for an AAV vector plasmid and a combined AAV helper and packaging plasmid (pDG/pDP) were produced and compared to their plasmid counterparts in terms of particle yield, packaging and transduction efficiencies and absence of prokaryotic sequences, for the production of ssAAV and scAAV vectors in HEK293 cells [10]. Viral vector preparations from MCs resulted in higher transgene-containing particles which pointed to better viral genome replication and packaging efficiency, and in the case of scAAV, resulted in viral particles with 30-fold higher transducing titers for HeLa cells [10]. Particularly for scAAV, replacement of both vector and helper plasmids with their relative MCs, resulted in diminishing the ampR sequence-containing particles from about 26.1% to below background levels relative to transgene-containing particles, whereas for ssAAV this was reduced from 2.5 to 0.004%.

The purity concerns expressed above are particularly critical in the case of non-viral vectors. Application of plasmid DNA vectors for SB-mediated transposition of transgenes for generating CAR-T cells have been dogged by poor transfection rates and high T cell toxicity [51]. The retroviral vector alternative is comparatively efficient but poses a low but serious safety concern regarding insertional oncogenesis and the theoretical risk of induced malignant transformation [52]. This led to the application of MCs for SB-mediated transposition which had earlier been reported in HeLa cells to result in improved stable transfection rates relative to plasmid-based vectors both while using an MC transposon donor as well as a complete MC-based mobilization system with transposon and SB-transposase from MCs [32]. Transfection of minicircle vectors for SB transposase and SB transposon in the form of CD19-specific CAR genes was successfully demonstrated in $CD8^+$ and $CD4^+$ T cells with a significant increase in transposition rate when compared to plasmid vectors for the transposase and transposon (4.4-fold). Furthermore, stable transgene expression was provided over multiple expansion cycles. Additionally, minicircles were also found to be less toxic to T cells in comparison to plasmids which together with the better transposition rate led to an overall improvement in yield of CD19-CAR-T cells [51]. CAR-T cells produced through MC vectors


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were comparable to those produced by lentiviral vector transduction in terms of proliferation, production of IFN-γ and IL-2 and in their cytolytic activity against CD19-expressing target cells as well as in an *in vivo* $CD19^+$ lymphoma xenograft model. This is expected to bolster the non-viral route for gene and cell therapy strategies since insertion site analysis of T-modified cells revealed that nucleotides in the vicinity of insertion sites had a near random frequency of occurrence when using MCs for transposition. Although insertions into generic regions were over the random expected frequency, they were still substantially fewer than those from LV integrations. Most importantly, MC-based SB transposition displayed a far better safety profile by coming closer to random insertion positions at genomic safe harbors, which was much higher than what was achieved with LV [51].

Earlier, it was shown that delivering the components of the SB system into mouse and human HSCs in the form of MCs, was more efficient and resulted in less electroporation cytotoxicity in comparison to plasmids. The result was that blood precursor cells could be stably modified in quantities that were sufficient to allow reconstitution of the hematopoietic system upon transplantation into mouse recipients [33].

Recently, the first DNA vaccine based on MCs was described [2]. The gene of interest was a hepatitis B surface antigen S2S, which was driven by the cytomegalovirus (CMV) promoter. The size of the DNA molecule was effectively reduced from 5737 bp in the initial plasmid pCMV-S2S to 3153 bp in the minicircle MC07.CMV-HBS2S. Testing of the immunogenicity of the plasmid DNA vaccine and its minicircle counterpart showed that injection of equimolar amounts of the plasmid or minicircle into B6 mice resulted in comparable fractions of $IFN\gamma^+$ CD8 T cells after *ex vivo* simulation of splenic CD8 T cells with specific peptides [2]. Efficient expression of the antigen molecule from the minicircles by the target eukaryotic cells ultimately balanced the loss of bacterial CpG motifs that may have been useful for stimulating the cell's innate immune response. One particularly important aspect to note here is that due to the reduced molecular size, a smaller dose of vaccine is sufficient to provide the necessary amount of gene copies and this precludes any potential DNA toxicity.

## TRANSLATIONAL INSIGHT

MC DNA is becoming increasingly important for clinical applications, in particular as a starting material for GMP production of viral vectors such as AAV [10] or cell-based immune therapies, for example against cancer [51]. Hence, in the near future, a process for High Quality Grade MC production [53] will be established. This High Quality Grade can be used for viral vector or RNA production, since full GMP is not necessary (depending upon the regulatory authorities responsible) for these applications [49,53]. A prerequisite for direct clinical use is GMP manufacturing of MCs according to regulatory requirements [1], even if the MC DNA is not directly injected in patients but for example used to modify cells *ex vivo* [51].

Currently, a MC version of the helper and packaging plasmid pDP2rs for AAV vector production, has been

successfully tested [10]. In advancing this important toolkit, MCs for AAV serotypes other than AAV2 are being developed (PlasmidFactory GmbH & Co. KG, Bielefeld, Germany);

In an ideal case, even the transgenic expression cassette needs to be free of CpG dinucleotides to minimize the risks of inflammatory responses even while using MCs [30]. In fact, Bazzani *et al.* place the focus entirely on the transgene expression cassette to be CpG-free, irrespective of the presence of a prokaryotic vector backbone, in order to obtain persistent expression in the murine lung [4]. However, simply the presence of such a backbone caused the expression to drop further drastically when the transgene cassette contained CpG dinucleotides while also showing a relationship with the CpG content in the backbone;

A significant application of MCs is their use as a non-viral alternative to integrating viruses for modifying somatic cells for the generation of iPSCs. MCs allow multiple reprogramming genes to be efficiently delivered in a single cassette. Whereas, this was first shown in hASC by Jia *et al.* [54], other recent developments are also being reported;

Another interesting application of MCs is the study of their therapeutic efficiency through transfection into cancer cells and subsequent silencing of oncogenes or expression of cytokines, as described by Gaspar *et al.* [34].

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## FINANCIAL & COMPETING INTERESTS DISCLOSURE

*The authors are employees at the company PlasmidFactory GmbH & Co. KG, Bielefeld, Germany, which holds the relevant patents on minicircle technology and a worldwide exclusive license on AAV vector technology (Kleinschmidt, DKFZ, Heidelberg, Germany).*



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