pEPI for Gene Therapy: Non-viral episomes and their Application in Somatic Gene Therapy

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Abstract

Nowadays, in most gene therapy trials virus based vectors are used because of their high efficiency. Nevertheless, safety risks like transformation of the cell by viral proteins, insertional mutagenesis, or innate immune reactions cannot be excluded. Basing on the idea of an ideal vector for gene therapy that is highly efficient but lacks these safety risks, non viral vectors systems represent an attractive alternative. With the construction of the non viral, S/MAR based vector pEPI at the end of the last century, a first step towards non viral gene therapy has been made. S/MAR based vectors do not contain any viral elements, do not integrate and show stable transgene expression in the targeted cell or organism. Within the last decade, S/MAR based vectors were further improved and modified, and find now broad application in basic research and also become more and more recognized in gene therapeutic and clinical trials.

Introduction

The development of an episomal vector

During the last decade many clinical gene therapy trials have taken place. Besidees beneficial results for the patients, also severe problems became obvious regarding safety aspects of the currently used technologies. Currently, a variety of episomal vectors of different origins find broad application in gene therapeutic approaches. Episomal vectors of viral origin benefit from their high transduction efficiency of most tissues and cells. Nevertheless, their successful application is limited by several safety issues. For example, adenooviral vectors were successfully used for gene therapeutic correction of Crigler Najjar (CN) disease in rats for more than 2 years [1]. These vectors can be produced easily, but bear the potential risk of random integration and toxicity [2,3]. Adeno-associated virus (AAV) based vectors have also been used in several clinical studies regarding cystic fibrosis, Parkinson’s disease, and haemophilia B [4-6]. AAV vectors display low toxicity, but low cloning capacity and rare integration events are limiting factors [7]. Episomal vectors that are of herpes simplex viral origin have a large cloning capacity and are capable of transducing neurons, myoblasts, retinal cells, and hepatocytes - but also bear the potential to integrate into the cellular genome [8-11]. Efficient and sustained transgene expression was also achieved with non-integrating lentiviral vectors. Even though the integration risk of these vectors is greatly reduced, loss of expression in non dividing cells and a remaining risk of insertional mutagenesis were observed [12]. Since episomal vectors of non viral origin lack the ability to transduce the target cell, they represent an alternative alternative to viral vectors. The naked DNA (plasmid DNA) needs to be transfected into target cells, and once delivered into nuclei these vectors exhibit different strategies for replication and maintenance. The Epstein Barr virus (EBV) derived plasmid replicon replicates once per cell cycle but requires expression of viral proteins (EBNA) bearing the risk of cellular transformation [13,14]. With the construction of the first human artificial chromosome (HAC) in 1997 a break through for gene therapeutic approaches was expected. The key advantages of HAC include their mitotic stability, non integrating maintenance, and large cloning capacity [15], while specialized delivery techniques are required [16]. Recently, HACs were firstly applied in a gene therapy approach using the mdx mouse model for Duchenne muscular dystrophy (DMD). In the two step approach, initially mesangioblasts from dystrophic mdx mice were genetically corrected with a HAC containing the human dystrophin genetic locus, and subsequently engrafted robustly into mice. A morphological and functional amelioration of the phenotype that lasted for up to 8 months after transplantation was observed and indicates a potential role of HACs in pre-clinical studies [17].

As outlined above, viral based vectors bear potential safety risks like insertional mutagenesis and may trigger innate immune reactions [2]. In consequence, viral vectors are used in gene therapy solely after a careful risk benefit analysis. Accordingly, non viral, autonomously replicating vectors are considered to be a safe alternative, but their application in gene therapy is nowadays strongly limited due to their low establishment efficiency [18]. Attempts to construct non-viral episomal vectors date back to the 1980s when ARS (autonomously replicating sequences) have been described in yeast [19]. The basic was to identify replication promoting sequences in mammalian cells and insert them into plasmids, very similar to the ARS assay in yeast. Thus, these “new” vectors would undergo the safety problems of viral vectors and should not lead to transformation of the recipient cell [18]. Yeast ARS are short sequences and represent binding sites for the ORC (origin recognition complex) [20]. The ARS isolated from yeast were found to promote replication when inserted into a plasmid [19]. When mammalian DNA was restriction digested and cloned into yeast plasmids, a multitude of sequences was found to promote replication in yeast. Transfected in mammalian cells however, the plasmids did not replicate autonomously and rather got lost from cells or integrated into the genome. These results suggested that some sequences of the mammalian genome somehow substitute ARS in yeast but do not

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act as origins of replication in mammalian cells. Insertion of putative mammalian origins of replication into plasmids resulted in a few rare cases in episomal maintenance [21,22]. Subsequent sequence analyses of various mapped mammalian origins of replication revealed no sequence homologies but rather a number of structural characteristics, such as long AT-rich regions, CpG islands, bent DNA and the presence of S/MAR (scaffold/matrix attached region) sequences [23,24]. S/MAR sequences are involved in a wide variety of biological processes in which compatibility to the nuclear matrix is of importance, including origin of replication function [25,26], modulation of gene expression [27], insulator function [28], and long-term maintenance of high transcription levels by counteracting DNA methylation [29,30]. In the late 1990 a non viral, episomal, and autonomously replicating plasmid was constructed in our lab: The S/MAR based vector pEPI replicates episomally and is mitotically stable over hundreds of generations in the absence of selection [31]. During the following years, pEPI attracted more and more attention. Based on the first prototype, inducible vector derivates were constructed, pEPI minicircle plasmids were developed, and facing to its potential application in gene therapy approaches, pEPI was improved for in vitro and in vivo applications. These issues will be discussed in detail in the following section, pointing out an exciting role for pEPI in basic research and future gene therapeutic applications (Table 1).

### Episomal S/MAR vectors

As mentioned above, the first vector shown to replicate autonomously in a variety of cell lines and retained in the absence of selection was the vector pEPI-1. The large T antigen of SV40 was replaced by a S/MAR sequence derived from the human β-interferon gene cluster [31]. Multiple AATATATTATA elements in this sequence serve as DNA unwinding elements (DUE) and allow stress-induced DNA duplex destabilisation of dsDNA [32]. In CHO cells, pEPI replicates at low copy numbers with 5-10 copies per cell [33] and is stably retained in the absence of selection for basically unlimited time [31]. Episomal maintenance of pEPI was observed in several other cell lines including HeLa, HEK293, and even human primary cells.

| promoter | modification | application | cell line/ organism | transfection method | reference |
|----------|--------------|-------------|---------------------|---------------------|-----------|
| pEPI-1   | CMV promoter (prototype) | in vitro | CHO-K1, HeLa, IMEF, HCT116, CD34+ cells, | lipofection | Piechaczek et al. [31] Jenke et al. [34] Papapetrou et al. [43] Tessadori et al. [39] |
|          |              | in vivo | pig embryos | sperm-mediated gene transfer | Manzini et al. [44] |
| pEPI-CAG | CAG promoter | in vitro | CHO-K1 | lipofection | Manzini et al. [48] |
| pEPI-AAT | α1-antitrypsin (AAT) promoter | in vivo | mouse liver, neonatal mouse liver, lungs, heart, brain, kidney | hydrodynamic injection, systemic injection of PEI DNA | Argyros et al. [45] Wong et al. [64] |
| pEPI-Ubc | human Ubc promoter | in vivo | mouse liver, neonatal mouse liver, lungs, heart, brain, kidney | hydrodynamic injection, systemic injection of PEI DNA | Argyros et al. [45] Wong et al. [64] |
| pEPIto   | hCMV/EF1P   | in vitro | HEK293, NIH 3T3 | lipofection | Haase et al. [49] |
|          |              | in vivo | mouse liver | hydrodynamic injection | |
| pEPI-TetON | TRE tight module | in vitro | CHO-K1 | lipofection | Rupprecht et al. [42] |
|          |              | in vivo | mouse liver | hydrodynamic injection | |

| backbone | modification | application | cell line/ organism | transfection method | reference |
|----------|--------------|-------------|---------------------|---------------------|-----------|
| pEPIto   | CpG depleted backbone | in vitro | HEK293, NIH 3T3 | lipofection | Haase et al. [49] |
|          |              | in vivo | mouse liver | hydrodynamic injection | |
| S/MAR based minicircle | lacking bacterial elements | in vitro | CHO-K1 | lipofection | Nehlsen et al. [54] Broll et al. [57] |
|          |              | in vivo | mouse liver | hydrodynamic injection | Argyros et al. [58] |

**Table 1**: Modifications of the prototype pEPI applied to the promoter and plasmids backbone.
In all tested cell lines the vector was mitotically stable in the absence of selection and occurred in an average copy number below 10 per cell [18], indicating a highly efficient replication and segregation. In established cells pEPI is associated with the nuclear matrix via the matrix protein SAFA and segregation occurs with the host chromosomes via hitchhiking (Figure 1) [34]. Although hitherto data are unavailable of preferential binding sites of pEPI to certain mitotic chromosomes, it may be possible that binding of pEPI is restricted to specific sequence elements [33,35]. Like the cellular genome, pEPI replicates once per cell cycle during early S phase. The origin recognition complex was shown to assemble at various regions of the episome [36], thus behaving as the initiation zone of genomic origins of replication [23] (Table 2).

After transfection and an initial selection phase, only a small percentage of vector molecules (1-5%) establishes stable as an episome, even though pEPI carries all cis-acting elements required for episomal replication and maintenance. This low establishment rate is one main limiting factor of pEPI for its use in gene therapy approaches. Therefore, it is most likely that epigenetic mechanisms are involved in the establishment process; a phenomenon that has also been reported for EBV-based vectors recently [37,38]. Once established pEPI localises in the interchromatin space (regions of less condensed chromatin) and is found to associate with nuclear speckles, a nuclear region involved in RNA processing. An association with early replicating foci that may be possible that binding of pEPI is restricted to specific sequence elements [33,35]. Like the cellular genome, pEPI replicates once per cell cycle in RNA processing. An association with early replicating foci that is stably retained over mitosis may explain the high mitotic stability of the vector. The establishment efficiency strongly depends on the nuclear compartment the vector reaches after transfection and the chromatin structure it thereby adopts. But when established pEPI behaves surprisingly non-dynamic throughout the cell cycle [33,39] and is associated with histone modifications typically found in active chromatin. Especially within the S/MAR region an accumulation of active modifications like H3K4me1 and H3K4me3 is detectable [40]. But regardless of successful establishment, only 30–70% cells containing pEPI as an episome display transgene (GFP) expression to a level detectable by FACS, suggesting that minimal transcriptional activity is sufficient to assure vector replication and maintenance. However, the expression profile of a single cell clone remains constant over numerous cell generations.

Modifications, Improvements and Next Vector Generations

The underlying mechanism of episomal replication and maintenance of pEPI is an active transcription running into the S/MAR. It has been demonstrated that whenever transcription running into the S/MAR was abrogated, i.e. by deletion of transgene, deletion of the promoter, or a termination signal between transgene and S/MAR, establishment failed and plasmids got lost from the cells or in some rare cases even integrated into the host genome [41]. Recently, in a proof of principle experiment an inducible vector was constructed in which transcription unit was under control of a tetracycline responsive promoter (TetON). Cells were transfected in the presence of doxycycline to ensure establishment. Subsequently, removing of doxycycline after establishment resulted in constant loss of vector molecules from cells. Furthermore, the pEPI-TetON system also works in vivo. Hydrodynamic injection of pEPI-TetON in mice and simultaneous administration of doxycycline resulted in a 5 fold increased transgene expression in mice liver. Despite these exciting results, the system demands further improvements since background expression of the transgene was also detectable in the absence of doxycycline [42]. These results provide evidence of an active transcription running into the S/MAR being essential for the episomal behaviour of the vector. Accordingly, modifications solely apply to vector backbone, insertion of additional, functional sequences, and insertion of diverse promoters (Figure 2).

Basically, the prototype vector pEPI-1 represents an almost ideal vector for gene therapy: Its episomal replication and maintenance has been shown in several cell lines [43] and has successfully been used for animal transgenesis [44]. Moreover, pEPI is completely based on chromosomal elements [31] and does not integrate into the cellular genome [41]. But pointing towards gene therapeutic applications, long-term transgene expression and episomal maintenance in every cell type are of immense importance. Subsequently, several endeavours were made to improve pEPI for in vitro and in vivo applications. It has been reported that early silencing of the CMV promoter in vivo is due to cytosine methylation within the CMV [45]. To circumvent early silencing in vivo, the CMV promoter of pEPI was replaced by a CAG promoter. The CAG promoter represents a synthetic hybrid promoter consisting of CMV enhancer element, chicken beta actin promoter

![Figure 1: (a) Simplified illustration of episomal maintenance. S/MAR sequences (blue) mediate binding of chromosomal DNA (loops) to the nuclear scaffold (brown). pEPI is bound to the nuclear scaffold via a SAF A – S/MAR interaction. Presumably, additional proteins are involved. (b) FISH staining of pEPI molecules (purple) attached to metaphase chromosomes (blue), using the hitchhiking principle to be segregated during mitosis.](image-url)
transgene modification application cell line/organism transfection method reference

| transgene  | modification     | application | cell line/organism          | transfection method      | reference                  |
|------------|------------------|-------------|-----------------------------|--------------------------|---------------------------|
| pEPI-EGFP  | EGFP             | in vitro    | CHO-K1, HeLa, IMEF, HCT116, CD34+ cells, mouse liver | lipofection              | Piechaczek et al. [31] Jenke et al. [34] Papapetrou et al. [43] Tessadori et al. [39] Rupprecht et al. [40] |
| pEPI-Luc   | luciferase       | in vitro    | U251                         | lipofection              | Argyros et al. [58]       |
| pEPI-Luc   | luciferase       | in vivo     | mouse liver, neonatal mouse liver, lungs, heart, brain, kidney mouse left hind muscle RIF-1 tumors | hydrodynamic injection, system injection of PEI DNA ultrasound-mediated gene transfer with microbubbles | Argyros et al. [45] Wong et al. [64] Li et al. [70] |
| IBAC-S/MAR-LDLR | LDLR         | in vitro    | CHO ldr(-/-)                  | lipofection              | Lufino et al. [59]       |
| S/MAR-bLCR-HBB  | beta globin microlocous cassette | in vitro    | MEL, K562                     | lipofection              | Sgourou et al. [60]      |
| pBoLuca1   | bcl-2            | in vivo     | mouse liver                  | hydrodynamic injection   | Wong et al. [64]         |
| pEPI/P1/PDX1 | pancreatic transcription factor pdx1 | in vivo    | rat liver                    | hydrodynamic injection   | Cim et al. [66]          |
| S/MAR:UbC/GDNF/ S/MAR | rat glial cell line-derived neurotrophic factor (GDNF) | in vivo    | rat brain                    | stereotactical injection | Yurek et al. [69]       |
| pEPI-b3a2  | hU6-bcr abl shRNA | in vivo    | K562                         | lipofection              | Jenke et al. [61]        |
| pEPI-RNAi  | hU6-HBV shRNA    | in vivo    | HepG2-2.15                   | lipofection              | Jenke et al. [62]        |

Table 2: Modifications of the prototype pEPI applied to the transgene

Figure 2: (a) Schematic illustration of the functional elements of pEPI: Expression of the respective reporter- or transgene (green), driven from an active promoter (turquoise) has to run into the S/MAR element (blue). When these preconditions are not given, S/MAR based vectors fail to establish episomally. In consequence they got lost from the cell or, in rare cases, integrate into the cellular genome. (b) Modifications of pEPI vectors solely apply to promoter, transgene and backbone.
sequences and rabbit beta globin 3' UTR sequences [46]. This promoter is described to be less prone to cytosine methylation and displays stable transcription in a variety of tissues in vivo [47]. Inserted into pEPI 1, increased eGFP expression levels for pEPI-CAG (pEPI 1 25%, pEPI-CAG 55%) were detected [48]. Likewise, a combination of CMV enhancer element and elongation factor-1 promoter (hCMV/EF1P) gave long term gene expression in vitro and in vivo [49]. Moreover, to avoid innate immune reactions, a reduction of the CpG motifs in pEPi backbone has proven to be promising. The CpG reduced vector pEPito showed improved establishment rates in vitro, and transgene expression driven from pEPi was stable up to 32 days post injection in vivo [49].

Preventing innate immune responses is one of the main challenges towards safe gene therapy. Thus, the design of a vector lacking any bacterial elements was a step forward. The so called minicircles were originally constructed with the use of recombinases [50-55], but are nowadays also commercially available [56]. Like pEPito, S/MAR based minicircles display enhanced transgene expression and sustained transgene expression in vitro and in vivo. Introduction of two Flp recombination sites into pEPI at either side of the promoter transgene S/MAR cassette led to the first S/MAR based minicircle. The subsequent induction of a Flp recombinase resulted in a recombination event and the emerging of two circular units: one containing the bacterial elements and one containing exclusively the promoter transgene S/MAR cassette [54]. S/MAR minicircles are maintained episomally with increased expression levels and establishment rates in vitro [57] as well as in vivo [58]. A new generation of the S/MAR minicircles with a truncated S/MAR element (~700 bp) displayed further improved stability and transgene expression in vitro [57]. An overview of the S/MAR based vector generations is given in Figure 3. Interestingly, the prototype pEPI 1 as well as next vector generations establish within a certain range of plasmid copies per cell, suggesting a stringent copy number control in the recipient cells. The contradicting observation of similar copy numbers and broad variations in expression levels leads to the suggestion that epigenetic features, chromatin structure, and nuclear localisation strongly influence the regulation of transcription from non-viral episomes.

Applications

In vitro

As discussed above, S/MAR based episomes have been demonstrated to function successfully in vitro and in vivo. For example, episomal maintenance in primary human fibroblast-like cells and at low levels even in human CD34+ cells has been demonstrated [43]. To ensure natural regulation an expression of the therapeutic gene, insertion of whole genomic DNA loci including all regulatory elements into the respective vector is of utmost importance for gene therapeutic applications. Recently, it has been shown that pEPI is not only capable of expressing one single transgene. In two approaches, cloning of genomic loci into S/MAR based vectors was impressively demonstrated. The genomic locus of the human low density lipoprotein receptor (LDLR) with 135 kb in size was cloned into a S/MAR based vector [59]. The iBAC-S/MAR-LDLR was maintained episomally for at least 11 weeks and excitingly was also capable to restore LDLR function completely when transfected into CHO ldlr(-/-) cells [59]. In a similar approach, Sgourou and colleagues were able to express beta globin at physiological levels by combining an S/MAR element with the beta globin microlocus cassette, including the locus control region of beta globin (betaLCR-HBB) [60]. However, besides the utilisation of S/MAR based vectors to express therapeutic genes, they are also suitable for expressing gene regulatory sequences. Following the idea of silencing viral genes, the bcr abl fusion gene was used as a first target for shRNA expressed by pEPI. Indeed, a significant decrease of bcr-abl gene expressing in K562 cells was detectable, while again the vector behaves like an episome in the absence of selection for at least four month [61]. In a next approach, shRNA targeting HBV replication associated gene expression was designed and cloned into pEPI. A significant reduction of HBV DNA content in HepG2-2.15 cells was detectable eight month post transfection [62].

In vivo

Five years ago, pEPI was firstly utilised in a fascinating in vivo approach. Vector DNA was delivered into female pigs using sperm mediated gene transfer (SMGT) with surprising results: Transgene (GFP) expression was ascertained in nine out of 12 modified pig foetuses (GFP) expression was ascertained in nine out of 12 modified pig foetuses with up to 80% of GFP expressing cells [44]. The high efficiency in this first in vivo approach indicated that pEPI is capable of adopting the respective epigenetic features needed for efficient establishment and propagation in the developing organism. But researchers were confronted with a much more complex situation when pEPI was firstly delivered into differentiated tissues. These approaches seem to be more challenging, as one of the main limiting factors is rapid silencing of transgene expression. As mentioned above, this silencing process was described to be due to cytosine methylation within the promoter sequence and often occurs within the first week post transfection [45,63]. The insertion of tissue specific promoters was a crucial step.

![Figure 3: Schematic depiction of pEPI vector generations](image-url)
to overcome this phenomenon. Cloning of a liver-specific promoter (alpha1-antitrypsin; AAT) into pEPI resulted in prolonged transgene expression in mice liver for up to 24 weeks [45]. Since it is known from _in vitro_ experiments that S/MAR based vectors stably establish in mitotically active cells, this situation was mimicked in the mice liver by a 70% partial hepatectomy.Surprisingly, both, the AAT as well as the CMV based vectors failed to establish in the regenerating liver and subsequently got lost [45]. Again, a similar behaviour was observed _in vitro_ when establishment efficiency of pEPI was determined without an initial selection phase after transfection (own observations). An elegant strategy for _in vivo_ selection was published recently [64]. The authors provided a survival advantage for transfected cells over non transfected cells by introducing the bcl 2 gene in a liver specific pEPI vector (pEPI AAT). Briefly, the physiological ligand FasL initiates cell death in hepatocytes when bound to the liver transmembrane protein Fas. This pathway may also be activated by binding of the agonistic antibody Jo2, while BCL2 in turn acts like decoy receptor for Jo2 and thereby inhibits apoptosis. The AAT-S/MAR-bcl-2 plasmid was delivered to mice liver using hydrodynamic injection. Despite constant Jo2 challenges, luciferase expression was detectable in mice liver for at last 12 weeks indicating maintained bcl-2 expression [64].

Recently, a further step towards _pre-clinical_ application was undertaken when S/MAR based vectors were injected into neonatal mice [3]. Vectors were administered via the superior temporal vein of mice at age 1.5 days. Transcription was driven either from an UbC or AAT promoter and plasmid DNA was injected in combination with a polyethylene (PEI) complex. Using this approach an efficient transfection of lung, brain, heart, brain, spleen, liver, and kidney was achieved. The expression profile for the UbC driven vector gave a gradual increase of luciferase expression peaking at days 11 12 and decreased by day 25 reaching baseline levels. When transcription was driven from the liver specific AAT promoter, a corresponding expression profile was observed, highest luciferase expression levels were detected at days 11 12, and dropped thereafter. As expected, the AAT-S/MAR vector gave tissue specific expression in the mid-abdomen region [3]. The attenuated expression of the transgene around day 20 is believed to coincide with the rapid proliferation of hepatocytes and the steep increase of liver weight [42], a phenomenon that has also been described after a partial hepatectomy [45]. Likewise, also minicircles harbouring liver specific promoters (AAT or UbC) were examined _in vivo_. Both, mini AAT-S/MAR and mini UbC S/MAR showed significantly increased transgene expression 24 h post delivery when compared to mini CMV-S/MAR. Expression levels remained elevated over a detected time slot of 92 days [58].

**Pre clinical applications**

Towards _gene therapeutic_ clinical applications of S/MAR based vectors, first studies have been published recently. Ferber and colleagues mediated ectopic expression of a pancreatic transcription factor (PDX1) in mice liver by introducing PDX1 into the livers. PDX1 was not only expressed in liver, but also capable of reversing streptozotocin mediated hyperglycaemia [65]. Basing on these results, the pdx1 gene was cloned into different S/MAR based vectors and delivered to rat liver, determining level as well as duration of transgene expression [66]. The original pEP1 PDX1 gave an expression peak at day 1 post injection but dropped significantly at day 3. In comparison, expression driven from AAT-PDX1-S/MAR lasted up to day three and dropped gradually afterwards. Additionally, a vector similar to the above described pEPito (reduced CpG motifs in backbone) was used. The pEPito AAT not only expressed PDX1 at a high level, but also mediated a pancreatic phenotype in the rat livers. Insulin 2 expression was detected in 66%, elevated up to 70 fold on day 14 in comparison to day 1 [66].

Various studies focused on the application of S/MAR vectors in other differentiated tissues than liver, like adult central nervous system, muscle, and tumours. For CNS studies plasmids were constructed in which the transgene is flanked by two S/MAR elements [67-69]. Yurek _et al._ [69] for example examined plasmid DNA delivery of a S/MAR UbC GDNF S/MAR vector coding for rat glial cell line-derived neurotrophic factor (GDNF) and found broad GDNF expression up to 400 % over endogenous expression levels for at least 3 weeks after post-delivery. An analogous vector, S/MAR UbC LUC S/MAR, was delivered in a complex with lysine 30mer peptides substituted with PEG. One single intrathecal injection resulted in stably expressed luciferase for up to one year in the striatum [68]. Supported delivery strategies into muscle were similar successful: Microbubble mediated ultrasound delivery resulted in strong expression levels in muscle tissue, while expression from control vectors lacking a S/MAR element was silenced within a month [70]. The outcome of these studies points towards an utilisation of optimised S/MAR vectors in combination with certain gene delivery strategies, being highly beneficial in future gene therapy applications. Additionally, S/MAR vectors may find further applications in the field of cancer research. As shown previously, delivery of a luciferase expressing S/MAR vector into RIF 1 tumours via ultrasound has been used successfully to monitor tumour growth in mice [70]. In future, the functional luciferase expression encoded by S/MAR vectors may, besides monitoring, play an important in tumour trafficking or even cancer treatment.

Since S/MAR based vectors exclusively consist of chromosomal elements, their episomal maintenance gives an advantage over the potential safety risks of viral vectors. Moreover, their chromosomal maintenance, long term expression, and high insert cloning capacity make them an attractive tool for _gene therapeutic_ applications. The progressions made in minicircle development and the construction of inducible vectors highlight the potential of S/MAR based vectors for a broad range of applications, although further improvements are necessary for future clinical applications.

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**Author Disclosure Statement**

The authors declare no competing financial interest.

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