

Review

Episomal Vectors: Principle, Utility, and Application

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Academic Editor: Takeshige Otoi

Special Issue: [Genetic Engineering in Mammals](#)

OBM Genetics

2025, volume 9, issue 4

doi:10.21926/obm.genet.2504317

Received: August 31, 2025

Accepted: November 12, 2025

Published: November 24, 2025

Abstract

An episomal vector is a plasmid- or virus-based vector that is present extrachromosomally in cells after transfection. Although it disappears during cell proliferation, it can exist in non-dividing cells, such as neuronal and muscular cells, and continues to express a gene of interest (GOI). Such episomal vectors are usually based on sequences from DNA viruses such as bovine papillomavirus 1 and Epstein-Barr virus. When cells are transfected with an episomal vector



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harboring a drug-resistance gene and subsequently cultivated in a medium containing a selective drug, the transfected cells would survive and continue to express the GOI. However, cultivating these cells in the absence of a drug may result in plasmid loss and reduced GOI expression. This seamless property of an episomal vector is especially advantageous for generating specific cells, as exemplified by induced pluripotent stem cells (which have been transdifferentiated after transfection of fibroblasts with reprogramming factors) without exogenous DNA. Moreover, the episomal vector has been improved using chromosomal S/MAR (scaffold/matrix attached region) derived from the β -interferon gene with episomal retention properties. This improved vector enables long-term expression of GOI, even in the absence of a selective drug. This property will be beneficial for its application in various scientific fields, including basic research (i.e., the generation of genetically modified animals) and gene therapy. This review describes the utility and applications of episomal vector-based gene expression systems.

Keywords

Episomal vector; non-integrating; long-term expression; S/MAR (scaffold/matrix attached region); SMGT (sperm-mediated gene transfer); genetically modified animals; oriP/EBNA-1; gene-of-interest; iBAC (infectious bacterial artificial chromosome)

1. Introduction

Recombinant DNA technology uses vectors, such as plasmids and viral vectors, to insert a gene of interest (GOI) into a host cell. These modified vectors are essential tools that have enabled a deeper understanding of gene regulation, led to the production of therapeutically important proteins like insulin, and paved the way for new vaccines and diagnostic tools [1, 2]. In this case, chromosomal integration of foreign DNA into the genome of a cell or organism is a prerequisite for this purpose (i.e., overexpression or knock-in of GOI or knockdown or knockout of the endogenous gene targeted), mainly when used for the continuous isolation of cells expressing heterologous proteins and for basic research on gene therapy. However, DNA insertions can have significant effects by introducing mutations, a process called insertional mutagenesis, or by triggering epigenetic silencing through *de novo* methylation, which can turn off the inserted gene or nearby cellular DNA [1, 2]. Furthermore, DNA insertion events can induce alternative splicing, aberrant transcription, and read-through transcription of endogenous genes by introducing new regulatory elements or disrupting existing ones. Thus, insertional mutagenesis poses a significant challenge for integrating vector-based gene therapy in humans.

In this context, non-integrating vectors, also referred to as “episomal vectors”, offer an attractive alternative, as they may be helpful as a potentially safer approach by avoiding insertional mutagenesis [3]. These episomal vectors, which are classified as plasmid- or virus-based, remain as non-integrating vectors in cells after gene delivery but disappear during cell proliferation because they are diluted with each cell division. On the other hand, episomal vectors can exist in non-dividing cells, such as neuronal and muscular cells, and continue to express GOI. This is a key advantage for gene therapy in these tissues. Episomal vectors are often based on viral DNA sequences from DNA

viruses like Epstein-Barr virus (EBV) and bovine papilloma virus 1 (BPV-1) because these viruses can naturally replicate as extrachromosomal elements within host cells [4-8]. When cells are transfected with an episomal vector harboring a drug-resistance gene and subsequently cultivated in a medium containing a selective drug, the transfected cells would survive and continue to express the GOI. However, cultivation of these cells in the absence of a drug may result in the loss of vectors and reduced GOI expression [9].

Researchers improved an episomal vector by using chromosomal S/MAR (scaffold/matrix attached region) derived from the human β -interferon gene (*IFNB1*) with episomal retention properties for sustained GOI expression [3, 10-14]. This improved vector enables long-term expression of GOI in the absence of a selective drug [15]. This property is beneficial for applications across various scientific fields, including basic research and gene therapy.

Currently, two vector types, viral and plasmid-based, are widely used in research and gene therapy. The former are classified into adenovirus (AV), lentivirus (LV), retrovirus (RV), adeno-associated virus (AAV), and Sendai virus (SeV) vectors [16]. These vectors can achieve efficient delivery both *in vitro* and *in vivo*, but can elicit immunologic responses, especially with AV vectors [16]. In contrast, plasmid-based vectors are characterized by their ease of manipulation, which leads to low immunogenicity. However, their primary limitation is low transfection efficiency compared to viral vectors [17].

In this review, we aimed to highlight the utility and application of an episomal vector-based gene expression system, especially emphasizing the potential of the non-integrating plasmid pEPI-1 and its derivatives (carrying S/MAR elements) to enable continuous expression of GOI *in vitro* and *in vivo*, as well as of the non-integrating LV vectors (NILVs) which exhibit stable transgene expression over time without loss of episomal viral DNA.

2. Non-Integrating Viral Vectors

Unlike natural viruses, viral vectors do not cause diseases because they are modified by replacing replication-related genes with therapeutic genes. For example, RV and LV vectors can be designed to prevent chromosomal integration into the host cell genome, creating non-integrating LV vectors for applications requiring transient or episomal gene expression. This is achieved through mutations in the viral integrase enzyme or by deleting key viral DNA sequences, reducing the risk of insertional mutagenesis and malignant transformation. While viral vectors traditionally produced in packaging cell lines such as HEK293 or A547 offer advantages in gene delivery, recent advancements in non-viral methods provide significant benefits in large-scale production and reduced immunogenicity. The following sections describe the use and properties of non-integrating viral vectors.

2.1 Non-Integrating AV Vectors

AV vectors have been widely used as vaccine candidates or potential vaccine candidates for the treatment of infectious diseases. They also offer ease of production, high titers, and high transduction efficiency into many cell types (including dividing and non-dividing cells) [18]. However, the application of AV vectors is limited by challenges, including poor targeting, which results in the vector infecting multiple cell types, and a strong immune response against the vector itself, which prevents repeated use [18]. More importantly, AV vectors do not integrate into the host cell's genome and instead remain as episomes in the nucleus, leading to transient expression of the

transgene [19]. AV vectors exhibit high transgene expression, but this expression is transient. Zheng et al. [20] modified a preexisting AV vector to contain 858 base pairs (bp) of retroviral elements, envelope sequence, and two long-terminal repeats (LTR) to extend the transgene expression. Experiments proved that this modified vector showed extended, but not “permanent”, transgene expression, which is helpful for clinical applications.

2.2 Non-Integrating LV Vectors

LV vectors are powerful gene delivery tools that naturally integrate their genome into the host genome of both dividing and non-dividing cells through transduction. They can transfer large complex sequences into target cells without subsequent silencing of the transduced sequences [21]. This indicated that long-term GOI expression could be achieved using this system. In contrast, LV vectors can induce mutagenesis and cell transformation. Non-integrating LV vectors with high transfer efficiency in both dividing and non-dividing cells and no risk of insertional mutagenesis were developed to improve the safety of LV vectors [22]. For example, Apolonia et al. [23, 24] constructed non-integrating LV vectors by mutating *cis*-acting sequences that interact with integrase (*att* sites) or by mutating specific residues in integrase in different domains. The resulting mutant vectors are referred to as integrase-deficient LV vectors (NILVs), which are efficiently produced and in which mutations do not affect infectivity. Notably, differentiated muscle cells infected with NILVs showed stable transgene expression over time without the degradation of episomal viral DNA, unlike dividing cells in which episomal viral DNA is degraded. NILVs are used in applications like vaccinations, gene repair, and cancer therapies where sustained, non-permanent expression is desired [24].

Terskikh et al. [25] constructed a self-inactivating LV vector encoding green fluorescent protein (GFP) cDNA and introduced it into mouse bone marrow cells (BMCs). After 7–16 months, BMCs from these primary mice were transplanted into secondary female recipients. The results showed that LV sequences were exclusively present in the primary cells of the hematopoietic tissues of mice, with no detectable presence in other tissues. These results demonstrate that self-inactivating LV vectors effectively target and integrate into the DNA of primary hematopoietic cells from mice, and that the subsequent transfer of these cells into secondary recipients did not result in the spread of the LV sequences to other tissues. Notably, Verghese et al. [26] inserted an S/MAR sequence into a NILV vector to enable stable exogenous gene expression in dividing cells. Consequently, this vector retained episomal transgene expression in these cells. Similar results were obtained when integrase-deficient LV vectors (NILVs) were used to incorporate the minimal S/MAR sequence [27].

2.3 Non-Integrating RV Vectors

Similar to LV vectors, RV vectors are another class that can naturally integrate their genetic material into the host genome. Non-integrating RV vectors have been developed to minimize the risks of insertional mutagenesis and off-target effects. This property offers potential advantages in gene delivery therapy, immunotherapy, and vaccinology. Yu et al. [28] constructed non-integrating RV vectors (IN-defective RV vectors) by introducing a point mutation into the IN catalytic core domain of the *IN* gene in a packaging plasmid derived from a Moloney murine leukemia virus (MoMLV)-based retroviral vector. The resulting IN-defective RV vectors efficiently transduced the target cells, but their gene expression was transient and lower than that observed with the

integrating vectors. IN-defective RV vector gene expression decreased to background levels after 10 days. Based on these results, IN-defective RV vectors may be helpful tools for efficient transient gene expression in non-dividing cells.

2.4 Non-Integrating AAV Vectors

AAV has a ~4.7 kilobase (kb) single-stranded (ss) DNA genome that is flanked by two inverted terminal repeats (ITRs) and contains the *rep* and *cap* genes [29]. The ITRs contain recognition signals for replication and packaging into functional virions, as well as for site-specific integration of the wild-type virus. The *rep* gene codes for replication proteins, while the *cap* gene codes for the capsid proteins that form the protective outer shell of the virus, which determines its tropism and ability to enter specific cells [29]. However, in recombinant AAV vectors used for gene therapy, the necessary viral genes for integration are removed, causing them to persist predominantly as non-integrating episomal concatemers in dividing and non-dividing cells [30]. Due to their relatively low immunogenicity compared to other viral vectors, AAV vectors are being used in clinical trials for ocular, neurological, metabolic, hematological, neuromuscular, and cardiovascular diseases [31].

2.5 Non-Integrating SeV Vectors

SeV vectors, which are viral RNA vectors, are among the most valuable tools for generating human iPSCs from various somatic cells (including fibroblasts) because they are non-integrating and can be efficiently removed once reprogramming is complete [32]. This renders the process safer for potential clinical applications, such as generating iPSCs that are free of viral and transgene DNA.

SeV vectors are well-known for their high transduction efficiency [33]. This efficiency is partly due to SeV vectors' ability to deliver genes to a broad range of host cells, including non-dividing cells. Additionally, they can be supplied with a short contact time, resulting in high gene expression.

2.6 Infectious Bacterial Artificial Chromosome (iBAC) Vector

Wade-Martins and colleagues [34] constructed a new vector referred to as iBAC, which is based on the herpes simplex virus type 1 (HSV-1) amplicon vector, enabling the delivery and expression of large DNA fragments, specifically >100 kb [34-37]. This new system overcomes the size limitations of earlier amplicons. It provides an expression system for large genomic regions, with advantages including minimal toxicity and a high transgene capacity, making it useful for gene therapy and other research applications [38]. It is possible to produce high-titer HSV-1 amplicon stocks without helper virus contamination by using a helper virus-free packaging system that employs a defective HSV-1 genome on a BAC scaffold that lacks the necessary packaging signals [39]. A typical iBAC map is shown in Figure 1. In this vector, EGFP expression is driven by a promoter from the HSV-1 *ICP4* gene (pIE4/5) to track vector delivery. The iBAC vector can be used to clone genomic DNA fragments using its unique *Bam*HI site. It allows the creation of a genomic DNA library in which each vector contains a different large genomic region. This is helpful for researchers creating genomic libraries for genome mapping, studying gene regulation, or sequencing large DNA segments. Notably, Wade-Martins and colleagues have demonstrated the usefulness of the iBAC system, which allows for more physiologically relevant and regulated gene expression of genomic DNA loci [35, 37] and delivers a locus to allow correct alternative splicing [36]. Thus, iBAC vectors have significant

advantages over traditional cDNA-based gene expression systems, which typically rely on strong, artificial promoters, leading to overexpression of a single splice variant and thus fail to capture the natural complexity of gene expression [38].

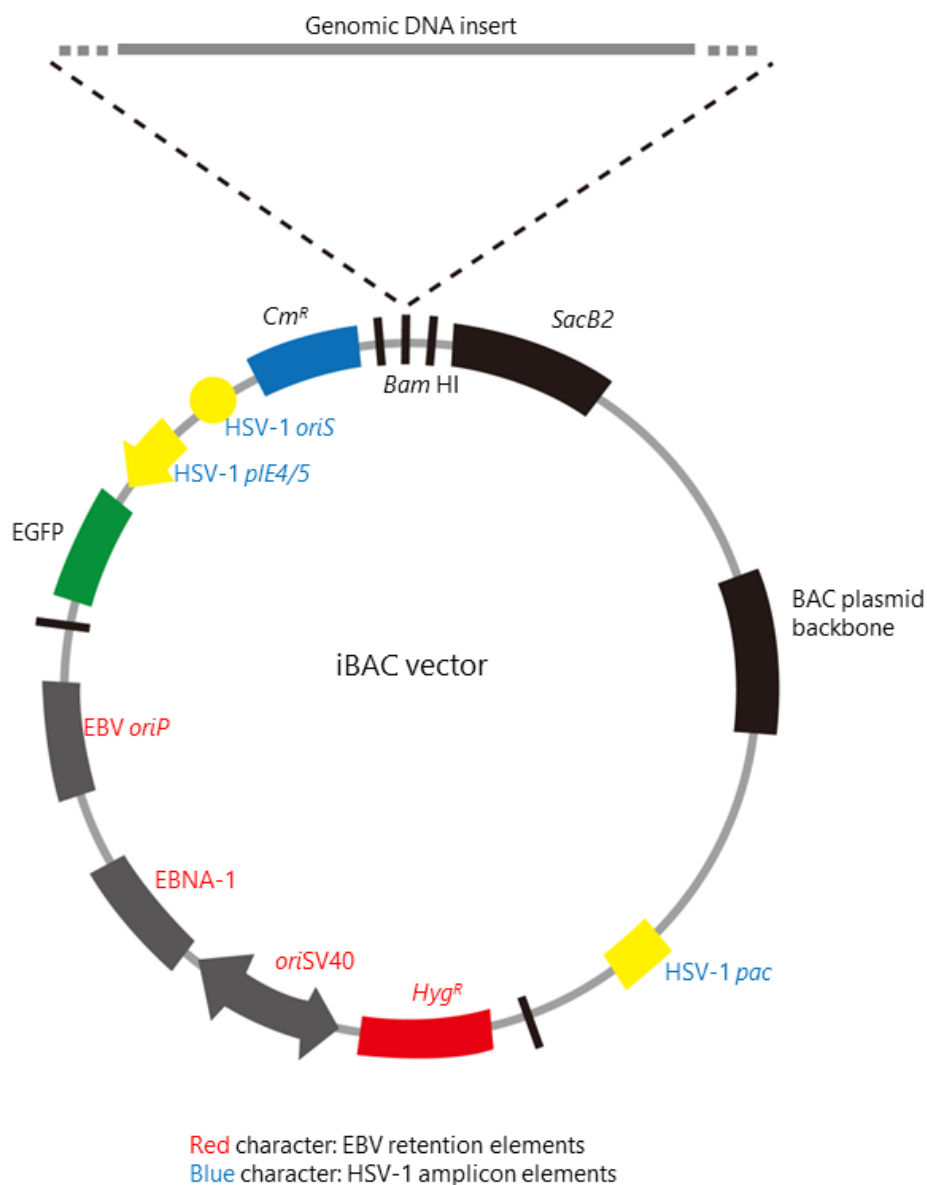


Figure 1 Schematic illustration of the iBAC library. The iBAC vector has genomic DNA fragments for packaging into herpes simplex virus type 1 (HSV-1) amplicon vectors, extrachromosomal retention, and bacterial replication. Genomic DNA fragments were cloned into the unique *Bam*HI site. This illustration was made in-house based on the paper of Lufino et al. [37]. *SacB2* (~1.4 kb), gene encoding the levansucrase enzyme, whose enzyme's activity is exploited in BAC library construction; HSV-1 *pac* (~90 bp), *pac* (or packaging signal) of HSV-1 to enable vector packaging; *Hyg^R* (~0.7 kb), gene conferring resistance to the antibiotic hygromycin B; *oriSV40* (~60 bp), origin of replication for SV40; EBNA-1 (~160 bp), Epstein-Barr virus nuclear antigen-1; EBV *oriP* (~1.7 kb), Epstein-Barr Virus origin of plasmid replication for long term vector retention in selected clones; EGFP (~0.7 kb), enhanced green fluorescent protein cDNA to track vector delivery; HSV-1 *pIE4/5*, the strong promoter/enhancer region (*pIE*) (~70 bp) of

the infectious lytic protein 4 (*ICP4*) gene in *HSV-1* to enable tracking of vector delivery and vector titration; *HSV-1 oriS* (~90 bp), DNA sequence in the *HSV-1* genome that serves as a replication origin (*oriS*) where viral DNA replication begins; *Cm^R* (~0.66 kb), gene conferring resistance to the antibiotic chloramphenicol.

3. Non-Integrating Non-Viral Vector

Several types of self-replicating non-viral vectors can be maintained extrachromosomally. These vectors contain components derived from (1) EBV elements [*oriP*/EBV nuclear antigen-1 (*EBNA-1*)], (2) BPV-1 elements [specifically the origin of replication (*ori*) and the *E1* and *E2* genes], or (3) genomic components (S/MAR region derived from human β -interferon gene). In the following, the major nonintegrating plasmid systems, such as *oriP*/EBNA-1-based episomal plasmids and S/MAR-based plasmids, will be mentioned.

3.1 Non-Integrating *oriP*/EBNA-1-Based Episomal Plasmids

Of these non-viral vectors, engineered EBV-based *oriP*/EBNA-1 vectors are frequently used as episomal vectors that persist in the nucleus as multi-copy episomes [40]. The 1.7-kb *oriP* region of the EBV genome allows recombinant plasmids to replicate and be stably maintained in human cells [41]. The *EBNA-1* gene is also an essential factor for the replication and maintenance of the EBV genome in infected human cells [41]. By binding to *oriP*, EBNA-1 promotes replication, nuclear transfer, and chromatin binding of *oriP*-bearing episomes to chromosomes/chromatin, and positively regulates transcription [42]. Thus, EBV-based *oriP*/EBNA-1 vectors enable efficient gene transfection, strong expression of exogenous genes, and the long-term maintenance of episomes in mammalian cells. Notably, the EBNA 1 protein can affect human cells by promoting their survival and immortalization through several mechanisms, including reducing apoptosis and increasing tumorigenicity [43, 44].

A typical map of the EBV-based *oriP*/EBNA-1 vector is shown in Figure 2. This vector contains expression units, such as antibiotic resistance gene expression units, which enable transfected cells to survive and proliferate in the presence of antibiotics. As long as continuous selection with the selective drugs is performed, it is possible to conduct functional analyses of genes and explore therapeutic genes to treat diseases like cancer [45-47].

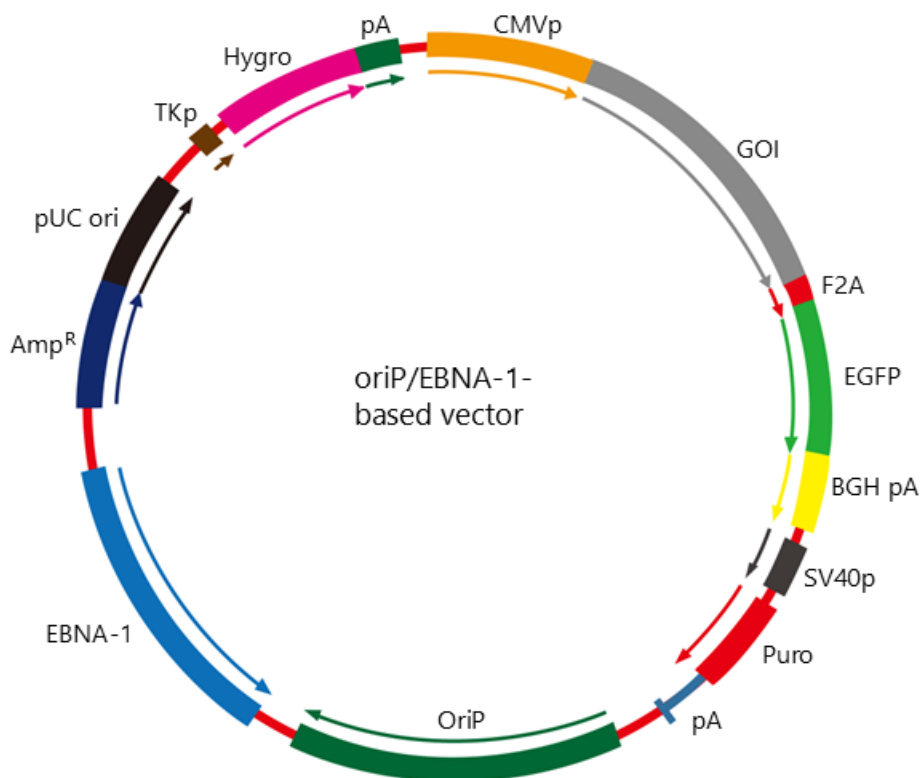


Figure 2 Schematic illustration of oriP/EBNA-1-based vector. CMVp (~0.6 kb), cytomegalovirus promoter; GOI, gene-of-interest; F2A (39 bp), 2A regions of foot-and-mouth disease virus; EGFP (~0.7 kb), enhanced green fluorescent protein cDNA; BGH pA (~215 bp), bovine growth hormone poly(A) signal; SV40p (~0.32 kb), SV40 promoter; Puro (~0.6 kb), puromycin resistance gene; pA, poly(A) signal; OriP (~1.7 kb), origin of replication for the Epstein-Barr virus (EBV); EBNA-1 (~0.16 kb), EBV nuclear antigen-1 (as replication transactivator of EBV); Amp^R (~1.7 kb), ampicillin resistance gene; pUC ori (~2.7 kb), replication origin; TKp (~0.1 kb), thymidine kinase promoter; Hygro (~0.7 kb), hygromycin resistance gene. This figure was drawn in-house, based on the figure shown in the paper of Li et al. [48].

3.2 pEPI-1 and Its Derivative as Ideal Vectors Showing Long-Term Expression in the Absence of a Selective Drug

The existing available vectors often silence the integrated transgene after transfection into host cells through unpredictable interactions with the host genome, resulting in an evident cessation of GOI expression. An ideal vector is genetically stable, safe to handle, nontoxic to host cells, highly efficient at transducing cells, and allows long-term GOI expression in the absence of selection. Lipps and colleagues [10] first constructed a non-viral episomal expression vector, termed pEPI-1 (Figures 3A, B), where the viral gene encoding the SV40 large T-antigen was replaced by a chromosomal S/MAR derived from the human β -interferon gene (*IFNB1*). This allows the vector to replicate episomally (outside of the main chromosomes) with high mitotic stability, enabling long-term, non-integrating expression of other genes within the same vector. Notably, there is no translation termination signal, such as a polyadenylation sequence, between *EGFP* cDNA and S/MAR (Figure 3B). This can result in a longer, non-functional mRNA transcript. S/MAR efficiently anchors

chromatin loops to nuclear matrix proteins to generate structural and functional chromosomal domains within the nucleus and has been successfully used for sustained gene expression through successive rounds of cell division ([13]; Figure 3A).

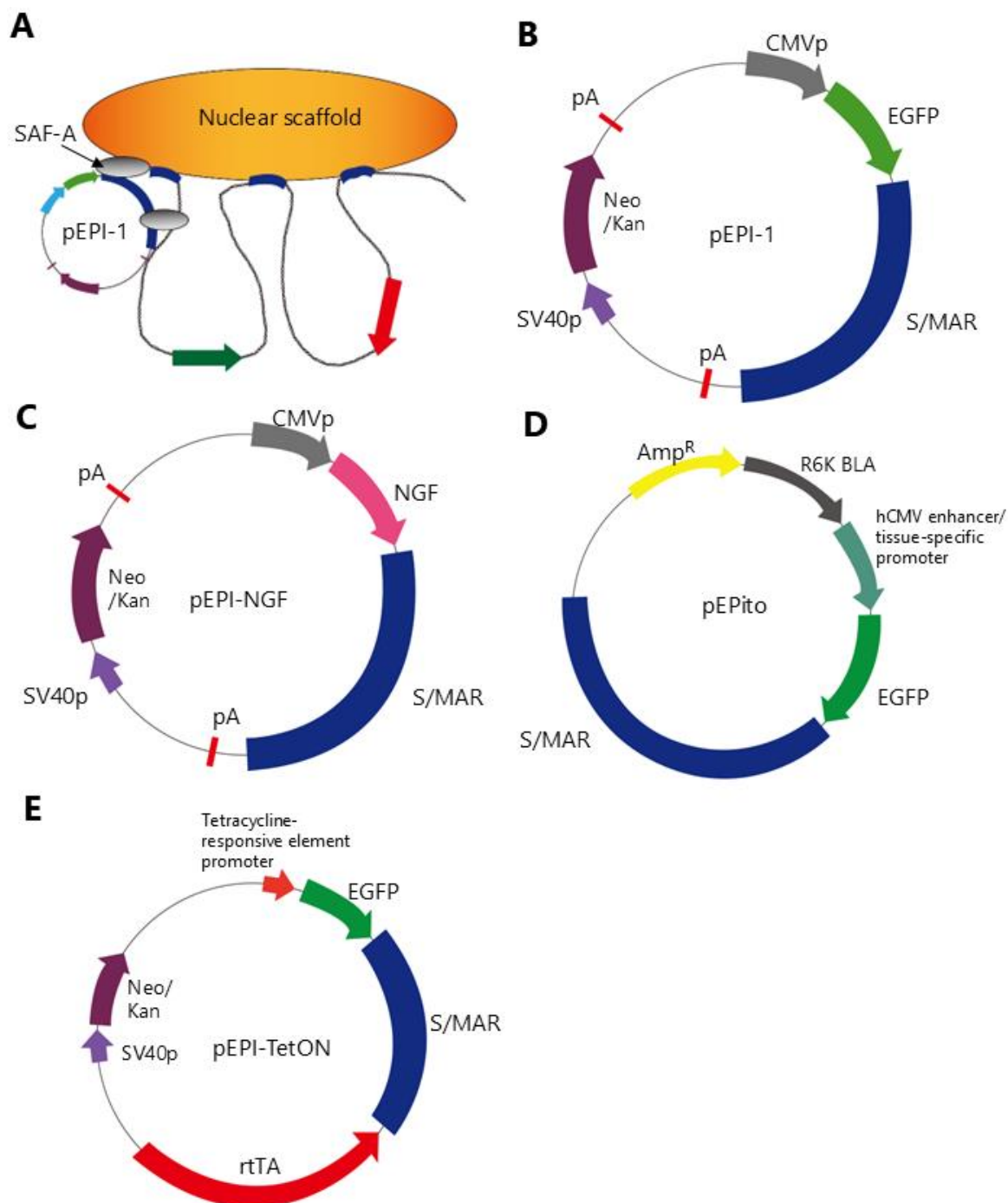


Figure 3 The S/MAR-based episomal vector pEPI-1 and its derivatives. **A.** Illustration of the maintenance of the S/MAR-based episomal vector pEPI-1 during cell proliferation. S/MAR sequences (blue) mediate the binding of chromosomal DNA (loops) to the nuclear scaffold (brown). pEPI-1 binds to the nuclear scaffold via scaffold attachment factor A (SAF-A; a protein that binds to S/MAR elements)–S/MAR interaction. Additional proteins are presumably involved in this interaction. **B-E.** Maps of (B) pEPI-1, (C) pEPI-NGF, (D) pEPito, and (E) pEPI-TetON. The vectors shown in (C)-(E) were derivatives of

the prototype vector pEPI-1. pEPI-NGF contained a gene encoding the truncated rat nerve growth factor (NGF) receptor gene 5' to the S/MAR sequence. pEPito represents a derivative of pEPI-1 with a CpG depleted backbone. pTetON contains a Tet-inducible element in which transgene expression is under the control of a tetracycline-responsive promoter. Amp^R (~1.7 kb), ampicillin resistance gene; R6K BLA (~1.6 kb), ~0.7 kb for the plasmid R6K ori + ~0.9 kb for the ampicillin resistance gene (bla) cassette; SV40p (~320 bp), SV40 promoter; rtTA (~1 kb), reverse tetracycline-controlled transcriptional activator; pA, poly(A) signal; hCMV enhancer, human cytomegalovirus (CMV) enhancer (~0.7 kb); EGFP (~0.7 kb), enhanced green fluorescent protein cDNA; Neo/Kan (~0.8 kb), resistance cassettes containing dual promoters to confer kanamycin resistance in bacteria and G418 resistance in mammalian cells; CMVp (~0.5 kb), immediate early promoter of human CMV; S/MAR (~2 kb), S/MAR region of the 5' area of the human interferon- β gene. These figures were drawn in-house based on the figure presented by Hagedorn and Lipps [13].

The pEPI-1 vector, which replicates episomally in Chinese hamster ovary (CHO) cells, is retained with a low, stable copy number (5–10 copies per cell) and is maintained in the absence of selection for hundreds of generations, a key feature for long-term gene expression [10]. Jenke et al. [11, 15] improved the vector by inserting a gene encoding a truncated rat nerve growth factor (NGF) receptor under the control of the CMV-promoter into pEPI-1. The resulting vector is termed pEPI-NGF (Figure 3C). The authors found that the CMV promoter in an episome was not silenced by cytosine methylation, thus enabling long-term expression of transgenes in the absence of selection [15]. Notably, Papapetrou et al. [49] demonstrated that in some lines (such as murine erythroleukemia cell line, MEL) expression of GOI (EGFP) from pEPI-1 is silenced by histone deacetylation, even though the vector remains episomal. This suggests that pEPI-1 does not behave uniformly across all cell types.

3.2.1 S/MAR Element Enabling GOI Expression at Physiological Levels

S/MAR-based vectors have been further improved and modified. They are now broadly applied in basic research and are increasingly recognized in gene therapy and clinical trials [13]. Lufino et al. [37] developed a derivative of iBAC by inserting a human S/MAR element to provide efficient vector maintenance and regulate the gene expression of GOI at physiological levels. This system is referred to as the iBAC-S/MAR vector (Figure 1). This vector is capable of infectious delivery and retention of large genomic DNA transgenes (135 kb; carrying the human low-density lipoprotein receptor (*LDLR*) genomic DNA locus). When CHO cells (lacking the *LDLR* gene) were transfected with this vector, cells with low-copy episomal components persisted for more than 100 cell generations without selection. Expression studies demonstrated that this vector completely restored LDLR function in those cells to physiological levels and that this expression can be repressed by ~70% by high sterol levels, recapitulating the same feedback regulation seen at the endogenous *LDLR* locus. This vector system overcomes the main problems associated with vector integration and unregulated transgene expression. Furthermore, Peruzzi et al. [50] constructed iBAC vectors carrying a 143 kb microtubule-associated protein tau (*MAPT*) or 135 kb α -synuclein (*SNCA*) locus, both of which are known to play central roles in neurodegenerative disorders. They found that cells transfected with these vectors

retained faithful gene expression, concluding that this system provides a novel method for analyzing genes associated with neurological disease. Sgourou et al. [51] developed a model to study tissue-specific gene expression using a construct containing the β -globin locus control region (β LCR) and the β -globin gene (*HBB*) on an episomal plasmid. This combination, referred to as β LCR-HBB, was placed within an *IFNB1*-S/MAR-based plasmid. The β LCR-HBB plus S/MAR combination constructs provide stable and reproducible expression of β -globin at physiological levels in a copy-number-dependent manner.

3.2.2 S/MAR-Based Vectors Showing Tissue-Specific Expression of GOI

A key challenge in reproducible gene therapy is achieving sustained, tissue-specific GOI expression. This was partially dissolved in a tissue-specific expression cassette. Haase et al. [52, 53] improved the expression of pEPI-1, referred to as pEPito (Figure 3D). This vector is a smaller, improved version of the pEPI-1 plasmid, which was designed to overcome issues with its predecessor. It was modified to have a single transcription unit and a 60% reduction in CpG motifs, which results in higher transgene expression and more stable expression in living organisms compared to pEPI-1. Furthermore, pEPito can be engineered to exhibit tissue-specific expression of GOI. For example, the constitutive promoter in pEPI-1 can be replaced by the tumor-specific alpha-fetoprotein (*AFP*) promoter or the muscle-specific smooth muscle 22 (*SM22*) promoter. Incorporating a human CMV enhancer element can significantly boost expression levels from tissue-specific promoters, which helps compensate for their inherently lower activity. This treatment allows for higher tissue specificity with fewer undesired side effects, which is beneficial for gene therapeutic approaches aiming for long-term transgene expression *in vivo*.

3.2.3 S/MAR-Based Vectors Conferring Regulated Expression of GOI

GOI expression is controlled by a tetracycline-responsive promoter, activated explicitly by the presence of doxycycline (DOX). This activation is mediated by a tetracycline transactivator protein (rtTA) that binds to the promoter only in the presence of DOX, thereby activating transcription [54]. This regulated gene expression system is known as the tetracycline-controlled transcriptional activation (TetON) system. Rupprecht et al. [55] constructed an inducible pEPI-1-based vector system referred to as pEPI-TetON (Figure 3E) by inserting an element containing the TetON system into the pEPI-1 vector. Stable cell transfectants were obtained by transfecting with pEPI-TetON and culturing in the presence of DOX. However, removing DOX after establishment led to transcriptional silencing of the gene linked to the S/MAR, resulting in vector loss from the cells. This feature enables the removal of all vector molecules from cells upon demand. This inducible episomal non-viral vector system will find broad applications in gene therapy as well as in the reprogramming of somatic cells or the modification of stem cells.

3.2.4 Nano-S/MAR DNA Vectors

The previous S/MAR-based non-integrating vector can provide persistent mitotic stability over hundreds of cell divisions, resisting epigenetic silencing, and thereby enabling sustained transgene expression. The composition of the original S/MAR-based vectors has inherent limitations that can induce cellular toxicity. Based on this background, Bozza et al. [56] modified a previous S/MAR-

based non-integrating vector and named nano-S/MAR. These vectors are designed to be small and can drive high transgene expression while avoiding the risks of insertional mutagenesis and other vector-mediated toxicities. Notably, a similar modification was made by Wang et al. [57], who demonstrated that shortened S/MAR regions are sufficient for replication and maintenance of episomes in mammalian cells (CHO cells).

3.3 Mini-Intronic Plasmid (MIP)

The bacterial backbone (BB) sequences in canonical plasmid DNA can cause transgene silencing, significantly reducing expression levels over time [58]. In this case, the length of the BB, rather than the sequence of the BB flanking the expression cassette, is thought to be one of the significant factors contributing to transgene silencing. According to Lu et al. [59], transgene silencing begins to occur when roughly 1 kb or more of DNA is located outside of the central transcription unit, specifically between the promoter's start and the poly(A) signal's end, even when the BB sequences are replaced with random DNA sequences. Lu et al. [59] developed an alternative plasmid (MIP) in which the essential BB elements for plasmid replication and selection were placed within an engineered intron contained within the eukaryotic expression cassette. These improved plasmids showed higher transgene expression than mini-circle vectors carrying the same expression cassettes, both *in vivo* and *in vitro*. These improved plasmids will benefit future studies involving gene transfer and therapy.

3.4 Micro-Linear Vector (MiLV)

As described previously, the presence of BB sequences within traditional vectors limits their use for the expression of GOI. Wang et al. [60] constructed a MiLV containing only a gene expression cassette (devoid of BB sequences) using PCR to avoid these limitations. The new vector system was superior to traditional plasmids for gene therapy because it leads to significantly higher and longer-lasting gene expression both *in vitro* and *in vivo*. These advantages may be beneficial for future clinical gene therapy research compared to plasmids, which have limitations such as lower efficiency and short-term expression.

3.5 Cumate-Inducible (CuO) Enhanced Episomal Vector (EEV)

Mullick et al. [61] examined how the bacterial cumate and cymene operons could be used to control gene expression in mammalian cells, creating an inducible system known as the cumate gene-switch. In this configuration, the cumate repressor (CymR) binds to an operator site (cumate operator, CuO) located downstream of a strong constitutive promoter, blocking transcription of genes that follow (upper column of Figure 4A). The addition of the small molecule cumate relieves transcriptional repression by binding to the CymR repressor protein, causing it to detach from the operator sequence (CuO) on the DNA (lower column of Figure 4A). The CuO EEV comprises the EBV oriP origin, the EBNA-1 factor (for maintaining the episomal genome in a host cell for sustained transgene expression), and a CuO unit (Figure 4B). Under a continuous supply of cumate, low background expression, dose-dependent induction, and nontoxic transgene expression are possible. Furthermore, this system has no limits on insert size (unlike AAV vectors). CuO EEV were purchased from System Biosciences LLC (Palo Alto, CA, USA).

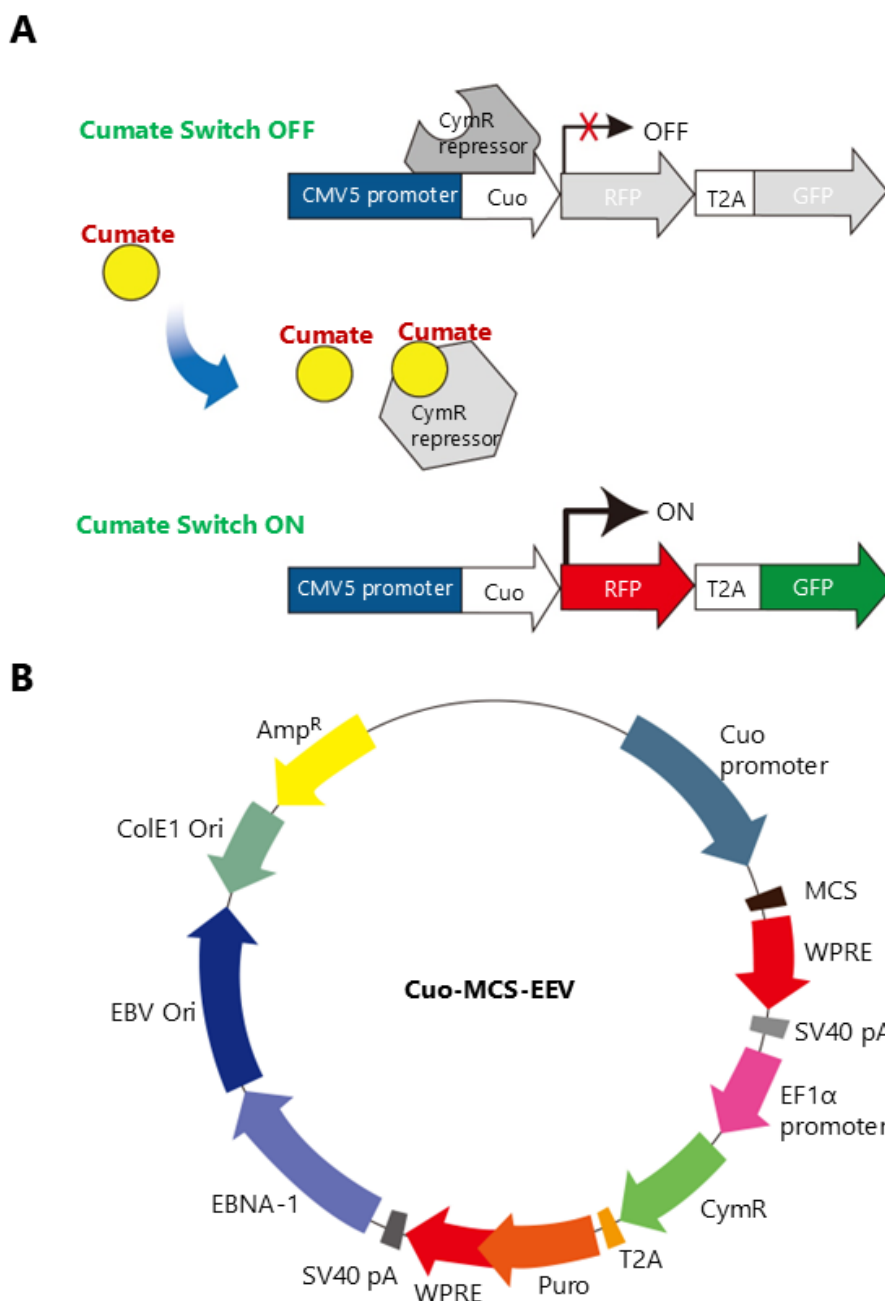


Figure 4 Schematic illustration of a cumate operator (CuO) enhanced episomal vector (EEV) conferring inducible and sustained GOI expression. **A.** Mechanisms of cumate-induced gene expression. The CuO promoter is a tightly controlled, inducible promoter that induces titratable gene expression in response to CuO accumulation. CMV5 promoter, an optimized version of the human cytomegalovirus (CMV) promoter that drives high-level gene expression; CuO, operator sequence; CymR, CymR repressor protein capable of binding to an operator site (CuO); RFP, gene coding for red fluorescent protein; T2A, coding for self-cleaving peptide derived from the *Thosea asigna* virus; GFP, GFP, coding for green fluorescent protein. **B.** Map of CuO EEV vector. CuO promoter (not specified), synthetic, cumate-inducible promoter made up of CMV5, a strong viral promoter and CuO sequences that are placed downstream of CMV5 and bind to CymR to silence expression; MCS, multiple cloning site; WPRES (~1.2 kb), a sequence to enhance the expression of GOI; SV40pA (~350 bp), simian virus 40

polyadenylation signal; EF1 α promoter (~2.1 kb), the promoter of elongation factor 1-alpha (*EF1 α*) gene involving in the elongation of polypeptide chains during protein synthesis; CymR (not specified), functions as the cumate-sensitive repressor for the CuO system; T2A (~60 bp), coding for self-cleaving peptide derived from the *Thosea asigna* virus; Puro (~600 bp), coding for puromycin resistance gene; Puro (~0.6 kb), puromycin resistance gene; EBNA-1 (~160 bp), coding for Epstein-Barr virus nuclear antigen-1; EBV Ori (~1.7 kb), Epstein-Barr Virus origin of plasmid replication for long term vector retention in selected clones; ColE1 Ori (~550 bp), ColE1 origin of replication, which is essential for initiating replication; Amp^R (~1.7 kb), ampicillin resistance gene.

4. RNA Virus-Based Episomal Vector (REVec)

REVec is a gene transfer system based on Borna disease virus (BoDV) that facilitates persistent intranuclear RNA transgene delivery without integration into the host genome [62]. REVec can transduce transgenes into various cell types and stably express them. However, this system lacks a mechanism to turn off transgene expression after REVec transduction. The same group [63] developed a novel REVec system, REVec-L2b9, in which transgene expression can be switched on and off using a theophylline-dependent self-cleaving riboswitch. Transgene expression from REVec-L2b9 was suppressed in the absence of theophylline but was induced by theophylline administration. Furthermore, Komatsu et al. [64] applied this system to induce human iPSCs from different somatic cell sources and demonstrated the highly efficient production of REVec transduction-mediated iPSCs. Notably, the introduction of a REVec carrying the myogenic transcription factor (*MyoD1*) gene into iPSCs led to successful transdifferentiation into skeletal muscle cells. Thus, the REVec system can be used as a versatile toolbox for stable integration-free iPSC modifications and transdifferentiation.

5. Application of Non-Integrating Vectors

5.1 Use of an Episomal Vector to Generate Genetically Modified Pigs

The genetic modification of animals is an invaluable tool in biotechnology and biomedicine. Although integrating vectors are currently being used for this purpose, they may lead to insertional mutagenesis and variable transgene expression. Manzini et al. [65] utilized the S/MAR-based vector pEPI-1 as a non-viral expression system to create genetically modified animals using the sperm-mediated gene transfer (SMGT) method. The SMGT method, initially developed in mice [66], functions consistently and efficiently in many animal species, including swine [67, 68].

According to Manzini et al. [65], washed sperm cells (1×10^8 spermatozoa) were first incubated with circular pEPI-1 plasmid (5 μ g of DNA) for 1 h at 17°C. The tubes were inverted every 20 min to prevent sperm sedimentation. The final 20 min incubation step was at room temperature, followed by heating (37°C) for 1 min immediately before the surgery. Laparoscopic insemination was performed with 5-mL aliquots per uterine horn containing 5×10^8 DNA-treated spermatozoa. Surgical dissection of the fetus was performed under anesthesia on day 70 of pregnancy. The presence of the pEPI-1 vector was detected in different tissues of 12 of the 18 fetuses analyzed by PCR. The vectors were retained in the episomal state. Expression of *EGFP* cDNA encoded by the pEPI-1 vector was confirmed in 9 of 12 genetically modified fetuses. Notably, in pEPI-1-positive

animals, all analyzed tissues expressed *EGFP* cDNA, with an average of 79% of cells positive. The high percentage of EGFP-expressing cells and absence of mosaicism suggest the usefulness of pEPI-1-based transgenesis in swine. Although germline transmission of the introduced transgenes remains to be elucidated, SMGT using an S/MAR-based episomal vector is an essential tool for creating genetically modified livestock such as swine (pigs) and bovines (cattle).

5.2 Use of Episomal Vectors to Engineer Hematopoietic Progenitor Cells

β -Thalassemia is a subgroup of inherited blood disorders associated with mild to severe anemia with few and limited conventional therapy options. For gene therapy of β -thalassemia, Lazaris et al. [69] constructed a non-viral, episomal vector pEP β -globin for the physiological β -globin gene based on two human S/MARs, enabling long nuclear retention, non-integration, and the β -globin replication initiation region (IR) to enhance replication and establishment. Successful nucleofection-based transfection was achieved in both K562 cells, a human erythroleukemia cell line derived from a patient with chronic myelogenous leukemia, and in CD34+ cells, hematopoietic stem and progenitor cells. The pEP β -globin vector will serve as a basis for the development of therapeutic nanoparticles, including extracellular vesicles, for gene therapy of β -thalassemia.

5.3 Use of Episomal Vectors to Engineer Human T Lymphocytes

The recombinant T cell-mediated therapeutic approach is now considered an efficient, fast, versatile, and safe genetic tool for combating tumors. Bozza et al. [70] used non-integrating, minimally sized DNA vectors to engineer human T lymphocytes. This vector platform contains no viral components and is capable of replicating extrachromosomally in the nucleus of dividing cells, providing persistent transgene expression in human T cells without affecting their behavior and molecular integrity. This technology can potentially be used to generate engineered T cells, referred to as chimeric antigen receptor (CAR)–T cells, which are modified to express a CAR to direct the patient's T cells to target and destroy cancer cells. Indeed, Bozza et al. [70] demonstrated that the nonintegrating minimally sized DNA vectors they produced exhibited enhanced antitumor activity *in vitro* and *in vivo* compared to previously described integrating vectors.

5.4 Use of Episomal Vectors to Generate iPS Cells (iPSCs)

The remnants of the introduced transgenes carrying reprogramming factors should be removed after iPSCs are established, as the transgenes incorporated into iPSCs may hinder proper differentiation and potentially compromise the clinical application of human iPSCs [71]. Based on this background, a variety of transgenes, as exemplified by non-integrating viruses, such as Sendai virus [32, 72, 73], and AV vectors [74], have been reported for the seamless creation of iPSCs.

The use of oriP/EBNA1-based non-integrating episomal vectors is also ideal; these vectors disappear from transfectants when the drug-based selective pressure ceases. Yu et al. [75] first described the derivation of human iPSCs using oriP/EBNA1-based non-integrating episomal vectors. After removal of the episome, vector-free iPSCs, similar to human embryonic stem (ES) cells, have proliferative and developmental potential. Since then, vector-free iPSCs have been successfully generated using fibroblasts [76], normal and neoplastic bone marrow and cord blood mononuclear cells [77], human lymphoblastoid B-cell lines [78], amniotic fluid stem cells [79], newborn cord blood

(CB) mononuclear cells (MNCs) (CB MNCs) or adult peripheral blood (PB) MNCs [80, 81], freshly drawn blood [82], and urine-derived cells [83]. Thus, reprogramming with episomal vectors is a cost-effective and straightforward method for generating exogenous DNA-free iPSCs. The iPSCs generated by using these episomal systems are referred to as “episomal iPSCs” [71].

5.5 Use of a Suicide Gene Vector to Generate Exogenous DNA-Free Cells

As mentioned previously, episomal reprogramming is an easy, safe, and cost-effective method for generating transgene-free (or integration-free) iPSCs. However, this system sometimes involves integrating transgenes into the genome. Although spontaneous removal of episomal DNA from cells occurs during cell division [76], cells reprogrammed by this system continue to harbor the episomal vectors after 10 passages in culture and undergo chromosomal integration of these vectors relatively frequently [84]. Lee et al. [85] employed a suicide gene system in episomal vectors to rapidly remove the DNA footprint. In more detail, the cytosine deaminase (*CD*) gene from yeast was inserted into an episomal vector to create new episomal vectors (called “CD episomal vectors”). CD catalyzes the conversion of the substrate 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), which is a lethal drug [86]. When the CD episomal vectors were used to reprogram human fibroblasts into iPSCs, these vectors were successfully eliminated from the generated iPSCs as early as seven days after 5-FC treatment. This novel approach will enable rapid, easy isolation of exogenous DNA-free reprogrammed cells.

5.6 Use of Episomal Vectors to Screen cDNA Libraries

Episomal vectors such as EBV-based vectors replicate in both eukaryotic and prokaryotic cells. In other words, they can be easily ‘shuttled’ from one cell system to another. Construction of an episomal vector cDNA library involves isolation of mRNA, synthesis of double-stranded cDNA, cloning the cDNA into an episomal vector, and introduction of this recombinant vector into host cells, where the cDNA clones can be maintained as stable, extrachromosomal elements. These episomal vectors can readily be recovered in *Escherichia coli*. [47]. The key advantage of this method is that episomal vectors can transfer large amounts of DNA derived from expressed genes (exons only) and can be easily manipulated for large-scale expression or for screening in eukaryotic hosts.

To our knowledge, Margolskee et al. [87] and Belt et al. [88] were the first to demonstrate that this approach is practical for screening cDNA libraries. They constructed EBV-based cDNA expression vectors containing the oriP element and the *EBNA-1* sequence, and the hygromycin B-resistance gene (*HpH*). The results revealed that cDNA inserts in the EBV-based cDNA expression vector were efficiently and appropriately expressed in recipient cells, probably due to the presence of the EBV sequences in the vector. The ability to directly select for expression of very rare episomal clones and subsequently recover them is beneficial for cloning specific genes where hybridization and immunological screening methods are not applicable, but where an observable trait (phenotype) can be scored or selected in various cell lines.

5.7 Use of Episomal Vectors to Study the Molecular Mechanisms Underlying DNA Replication or Mutagenesis

As episomal vectors utilize cellular enzymes for replication and repair, they represent powerful tools for studying DNA replication and mutagenesis [46]. As mentioned previously, episomal DNA is maintained and replicated in cells through the action of host factors recruited by viral proteins such as EBNA1 to the viral origin of replication, oriP. EBNA1 binds to oriP, recruiting the cellular Origin Recognition Complex (ORC) (first identified in *S. cerevisiae* as a complex of proteins essential for the initiation of DNA replication) and the minichromosome maintenance protein complex (MCM) helicase, which initiates DNA replication once per cell cycle [89]. Host factors such as the human proteins Timeless (Tim) and Timeless-interacting protein (Tipin) are also involved in EBNA1-mediated DNA replication initiation. Both proteins are known to form a mutually protective complex that is essential for stabilizing replication forks and play roles in sister chromatid cohesion and chromosome segregation, assisting EBNA1's function (ensuring that viral DNA is segregated to daughter cells), preventing loss during cell division [90].

Concerning the involvement of episomal vectors for the analysis of mutagenesis, Calos et al. [91] first demonstrated that an SV40-based shuttle vector containing lacI genes (related to the *lac* operon) of *Escherichia coli* that allow replication and selection in both bacterial and mammalian cells. Transfected into mammalian cells, the gene experiences a high mutation frequency, approximately 1% per gene, which is several orders of magnitude higher than spontaneous mutation rates. This high mutation rate leads to base substitutions, deletions, and sometimes insertions in the host genome. Calos et al. [91] speculated that the transfection process itself triggers these mutations, as they occur soon after the DNA enters the nucleus. In 1984, Ashman and Davidson [92] demonstrated that high spontaneous mutation frequencies were found in the shuttle vector pSV2gpt sequences recovered from mammalian DNA, with deletions being the most common mutation type, suggesting that small deletions are a predominant mechanism of spontaneous mutation in mammalian cells. These findings emerged from experiments where the vector's genes were analyzed for changes after being passed through mammalian cells. Later, the same group [93] used a recombinant shuttle vector containing the entire BPV genome, sequences from pBR322, and the *Escherichia coli* Xanthine-guanine phosphoribosyltransferase (*gpt*) gene to transfect mouse C127 cells. When plasmid DNA extracted from the transformed mouse cells was analyzed in detail, most of the recovered plasmid DNA had gross rearrangements in their DNA with a high mutation frequency. These experiments indicate the vector's susceptibility to damage or the presence of a mutagenic cellular environment, limiting its use as a precise tool for studying mutation mechanisms. This also suggests the need for the development of improved shuttle vector designs or experimental conditions to reduce background mutation frequency for more accurate research.

5.8 Use of Episomal Vectors to Manipulate the Mammalian Genome Using Genome Editing Technology

The clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system (CRISPR/Cas9) is a powerful gene-editing tool for introducing genetic mutations into various cell types. Li et al. [48] tested whether the gene encoding humanized *Streptococcus pyogenes* (*Sp*)Cas9 and gRNA, when included in oriP/EBNA-1-based episomes, can generate vector-free

mutations in mammalian cells. A puromycin expression unit was added to the above vector to enrich successfully further transfected cells. Flow-activated cell sorting (FACS) showed a 26% reduction in tdTomato-tagged mouse iPSCs in the group transfected with an episomal vector targeting *tdTomato* compared with the non-transfected cohort.

Xie et al. [94] developed a highly efficient gene KO system in human iPSCs, called “episomal vector-based CRISPR/Cas9 system (epiCRISPR)”. This system enables the generation of insertion/deletion (indel) rates of up to 100%. In addition, the epiCRISPR system enables efficient double-gene KO and genomic deletions. To minimize off-target cleavage, Xie et al. [94] combined episomal vector technology with a double-nicking strategy and recently developed a high-fidelity Cas9 (HiFi Cas9). Thus, the epiCRISPR system offers a high-throughput genetic analysis of human iPSCs.

Pfromm et al. [95] investigated the immunogenicity of the Cas9 protein using immunocompetent retinal cells such as human microglia (IMhu) and ARPE-19 cells, a spontaneously arising retinal pigment epithelia (RPE) cell line with normal karyology. Transfection with a *SpCas9*-expressing plasmid resulted in the successful expression of the Cas9 protein in both cell lines. However, ARPE-19 cells (but not IMhu cells) exhibit a pro-inflammatory immune response (including the release of cytokines and other molecules that trigger and amplify inflammation). Moreover, the viability of ARPE-19 cells reduced after transfection with both *SpCas9*-expressing and control plasmids. These findings suggest the need to develop additional techniques to overcome the potential immune safety risks in future Cas9-mediated retinal gene therapies.

5.9 Possible Use of Episomal Vectors towards Preclinical Studies

Episomal vectors are used in clinical gene therapy, particularly for creating integration-free induced iPSCs and for gene delivery to produce high levels of therapeutic proteins, such as in the case of severe genetic diseases or cancer [3]. For example, iPSCs isolated from patients can be engineered to correct defects and subsequently differentiated into healthy, functional cells for transplantation. Additionally, integration-free iPSCs and their differentiated derivatives can be used for tissue reconstitution as a cell-based therapy for human diseases. In fact, they are now being used in preclinical studies for treating diseases of the liver, nervous system, eye, and heart, and in diabetes [96]. Unfortunately, early-stage studies suggest the need for rigorous quality control, improved cell survival post-grafting, and the development of technologies to monitor transplanted cells during the grafting period. Furthermore, possible immune rejection, genetic instability, and tumorigenicity must be resolved.

For cancer therapy, NILVs have been used to develop personalized cell-based immunotherapies, using engineered T-cells and natural killer (NK) cells to attack tumors and reduce graft immune rejection. For example, a study by Karwacz et al. [97] showed that NILVs carrying a hepatitis B virus (HBV) gene, when injected intramuscularly, stimulated prolonged systemic CD8⁺ T-cell production and antibody responses to the secreted HBV surface antigen, and have also been proven to be an effective anti-tumor therapy.

6. Advantages and Drawbacks of the Non-Integrating Episomal Vectors

Episomal vectors can express genes in a target tissue/organ without mutational insertion of these elements into host chromosomes. Of these vectors, NILVs have been widely used in pre-clinical

research settings [96]. Generally, these vectors have higher transduction efficiency than other plasmid-based vectors. However, NILV-transduced cells lose their non-replicating nuclear episomes during rapid cell division, limiting their use for long-term expression. Therefore, NILVs represent a valid option for low-proliferating cells such as mesenchymal stem cells (MSCs). Notably, Xu et al. [27] provided options that overcome this difficulty. They added a minimal S/MAR sequence (SNIL) to the NILV and successfully demonstrated that this approach is helpful in retaining episomal transgene expression in dividing cells. In general, non-integrating viral vectors, as exemplified by NILV, have higher immunogenicity than other non-integrating plasmid-based vectors. Furthermore, production of non-integrating viral vectors is costly and generally more challenging to scale up than the manufacturing of many non-viral vector systems.

oriP-based vectors are also widely used in cultured mammalian cells. They are transiently present in cells after transfection and subsequent incubation. However, GOI expression will cease when transfected cells are grown without selective agents. The transient nature of GOI expression is beneficial for the acquisition of iPSCs and other genetically modified somatic cells used in cell-based therapy, due to the low immunogenicity and reduced risk of insertional mutagenesis compared to genome-integrating vectors. However, long-term gene expression is not possible in this system because these episomal vectors lack chromosomal integration. In contrast, other plasmid vectors, as exemplified by S/MAR-based ones (which are also termed “stable episomal vectors”), were still present even after removal of the selective drug. This property is beneficial for researchers who intend to perform long-term GOI expression. Furthermore, S/MAR-based non-viral vectors have a high capacity for large gene inserts, allowing the delivery of entire genomic loci, which facilitates more physiological and regulated gene expression compared to typical viral vectors with limited capacity. However, in this case, it is always accompanied by the risk of eliciting immunogenicity. Furthermore, in some cases, the continuous expression of GOI may cause unwanted abnormalities in terms of cellular behaviour. In this context, controlled GOI expression systems, as exemplified by TetON or cumate-induced systems, may be ideal to avoid the risks above. Alternatively, the re-voicing of vectors may be possible with the addition of drugs (e.g., 5-FC) to the culture medium when a drug-sensitive episomal vector is used.

7. Conclusions

The primary advantage of episomal vectors is that they do not integrate into the host cell's genome, thereby avoiding the risk of insertional mutagenesis and potential cell transformation (cancer). Episomal vectors based on transient expression systems, such as oriP/EBNA-1-based vectors, are helpful when any exogenous DNA must be removed. On the other hand, persistent gene expression systems, such as S/MAR-based vectors, provide long-term stability, but require transgene expression regulation to prevent potential side effects. Episomal vectors strike a balance between transient and persistent expression.

Non-viral episomal vectors have a high capacity for large gene inserts, allowing the delivery of entire genomic loci, which facilitates more physiological and regulated gene expression compared to typical viral vectors with limited capacity. Furthermore, they generally elicit fewer innate immune responses and are less costly and easier to produce on a large scale than many viral vector systems. On the other hand, viral episomal vectors, as exemplified by NILVs, are now being employed as an alternative to genome-integrating systems for applications in clinical gene therapy due to their

ability to transduce both proliferating (dividing) and non-proliferating (non-dividing) cells with high efficiency. However, careful safety assessments are essential for NILVs due to the inherent risks involving viral components and the potential for off-target effects.

There are still challenges, including variable delivery efficiency across different cell types and the potential for epigenetic silencing of the transgene. Ongoing research is focused on optimizing vector design (e.g., using CpG-depleted "minicircle" DNA and specific chromatin-modulating elements) and delivery methods (e.g., nanoparticles and electroporation) to overcome these limitations and enhance their clinical suitability.

In summary, engineered episomal vectors represent a valuable, safer alternative to integrating vectors, moving toward broader therapeutic and clinical use.

Acknowledgments

We thank Kazusa Inada for her support with the in-house drawing of Figures. This study was partly supported by a grant (no. 19K06372 to M.S.; no. 24K13200 to E.I.; 23K19334 for S.W.; no. 22H03277 for I.S.; no. 23K09441 for N.K.; no. 23K09450 for Y.I.; no. 23K27097 to S.N.) from the Ministry of Education, Science, Sports, and Culture, Japan.

Author Contributions

M.S. and S.N. designed and drafted the manuscript; E.I., S.W., I.S., N.K., Y.I. and K.M. critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

AI-Assisted Technologies Statement

During the preparation of this work, the authors used Google Gemini in order to improve sentence structure and clarity, especially shown in Sections 5 to 7.

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