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An Alternate Method for Efficient Delivery of Catalyzing Enzymes

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Abstract

Improved cellular engineering tools have enabled scientists to modify genomic sequences at a new pace for a variety of applications. However, in order to create more accurate tools for site specific targeting and editing, improved delivery systems for modifying enzymes are required. These enzymes allow end users to disrupt, insert or edit genes utilizing sequence specific homology. Current applications require co-transfection of an engineering plasmid in tandem with the vector carrying the modifying enzyme. However, co-transfection can negatively affect transfection efficiencies as well as increase probability of randomly integrating DNA. Here we report the transient delivery of catalyzing enzymes via BacMam non-integrating virus at high efficiency. Baculovirus is a non-infectious delivery system that can transduce a variety of mammalian cells. We have shown improved delivery of NLS-Cre recombinase for the excision of regions in disrupted genes to enable expression. In addition, we have tested PhiC31 integrase delivery for the guided insertion of plasmid DNA to a specific locus. The transient expression provided by BacMam, coupled with ease of transfection, creates an ideal system for delivery of catalyzing enzymes in mammalian cells.

Keywords: Catalyzing enzymes; Cre recombinase; Phi C31 integrase; BacMam

Introduction

The genetic modification of mammalian cells permits increasingly complex engineering methods for dissecting unknown cellular and molecular mechanisms. These modifications can be enhanced by the use of catalyzing enzymes for site-specific action or delivery. Enzymes for the modification of genes require additional platforms for delivery and expression. However, these platforms are limited by the lack of efficient methods for gene delivery to facilitate downstream engineering via robust expression.

The most commonly used engineering methods require the use of plasmid DNA or a workhorse virus. Naked plasmid DNA has been utilized as a routine delivery method; however, it is limited by the availability of efficient transfection methods for a variety of cell types [1]. Additionally, DNA can be unpredictable due to its propensity to randomly integrate possibly disrupting additional unknown processes and creating genetic instability [2]. Viral vectors easily overcome the limits of cellular penetration by naturally breaching the cellular membrane. However, many widely used viral techniques, such as AAV or lentivirus; also integrate randomly into the host cell genome for stable and robust expression. Adenovirus, on the other hand, does not integrate into the host cell genome. Although attenuated, the use of adenovirus in mammalian cells requires further care and assembly within alternate mammalian cell lines.

BacMam is a baculovirus engineered to express genes in a mammalian host cell via the addition of a mammalian promoter. Although the baculovirus can transduce mammalian cells, the virus is non-infectious, creating an ideal expression system in terms of safety and efficiency [3]. The BacMam system has been used to express gene content in mammalian cells previously, however its use as an engineering tool remains untapped. Additionally, the BacMam system has been outfitted with Multisite Gateway technology (Figure 1A) for rapid generation of gene libraries with interchangeable elements. This enables users to readily exchange the expression cassettes containing various modifying enzymes or promoters with ease. In addition, large quantities of virus can be easily generated (Figure 1B) and stored stably at 4°C for extended periods of time.

There are multiple gene editing tools that require temporary and robust expression of transgenes. Characteristics of these methods make each editing tool more suited for a specific application. The commonly referenced Cre-loxP system is an ideal candidate requiring only temporary expression of Cre recombinase. The system relies on enzymatic recombination of DNA at two loxP sites, excising any DNA between the two elements [4]. Additionally, phage integrases have been harnessed as effective gene editing tools for specific site targeting in a variety of cells [5-7]. Phage integrases provide direct recombination to either "hot spots" within the mammalian genome [8] or specific pre inserted genomic sequences [9,10]. These modifying enzymes have been utilized as proof-of-concept for the delivery of catalyzing enzymes via BacMam. Our results indicate that the delivery of Cre recombinase and Phi C31 Integrase via BacMam yields results similar to, or better than DNA-mediated delivery of these enzymes. Coupling of gene editing tools with Baculovirus provides an ideal tool for transient expression of gene editing tools without causing off site effects of random integration.

Materials and Methods

Cloning and virus production

Genes of interest (GOI) were cloned into Destination vectors using Multisite Gateway® Technology. These expression vectors carried gentamicin resistance and GOI flanked with Tn7 sites for BacMid production. BacMids were assembled by the transformation of the expression vector into DH10Bac competent cells. BacMids were transfected into Sf9 cells to generate viral clones. The virus was further expanded via plaque purification. BacMid production, virus expansion and transduction were performed as previously described [11]. Clonal

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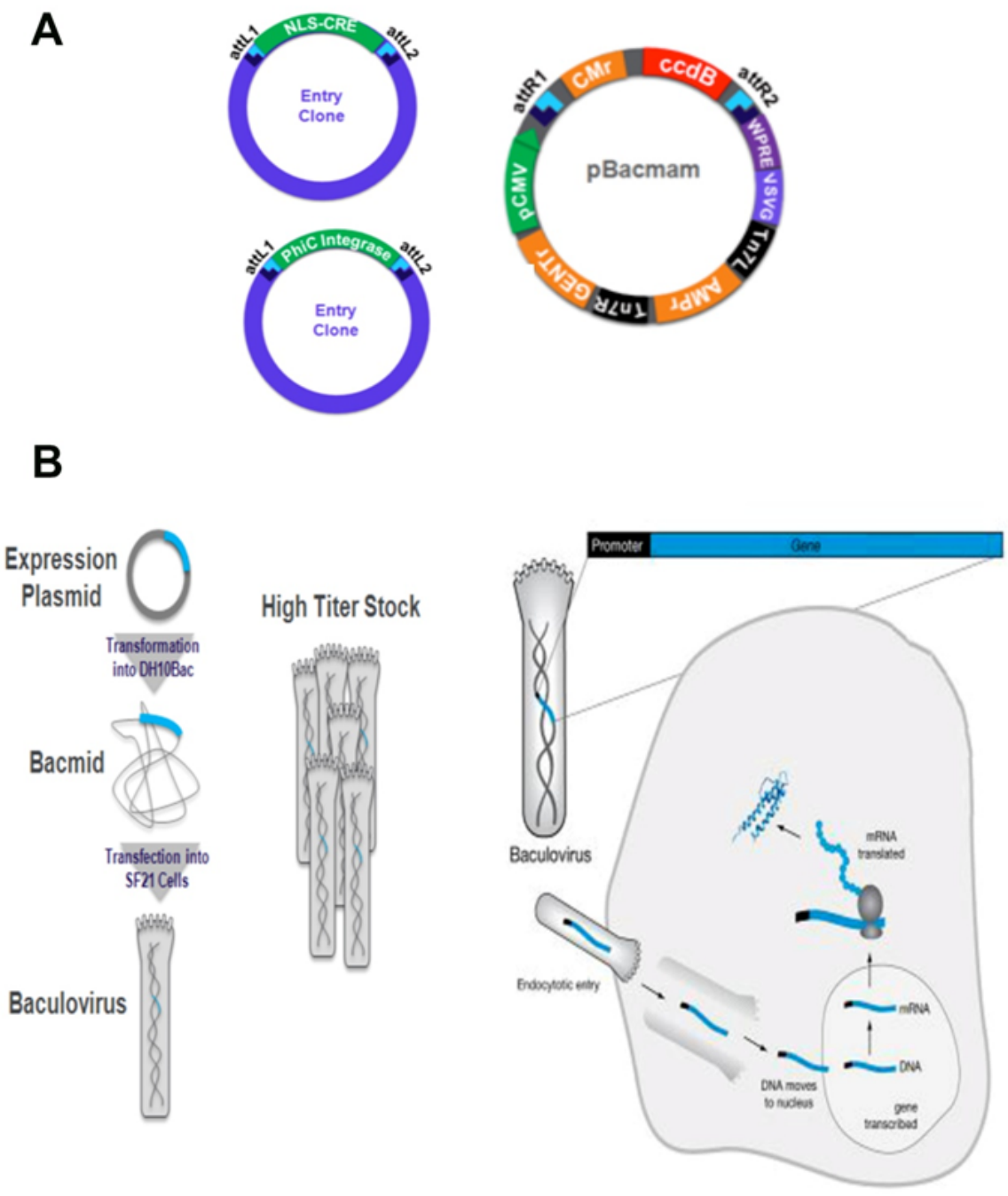

Figure 1: Generation of BacMam Expression Construct and Bacmid/Viral Production (A) The expression clone pBacMam is assembled using Multisite Gateway Technology. Entry clones containing genes of interest (GOI) are pre assembled in Entry vectors and added simultaneously via an LR Reaction. (B) Bacmids are produced from expression clones in DH10Bac cells and transfected into SF21 Cells for Baculovirus production. The virus is then used for expression of gene of interest in mammalian cells.

large-scale stocks were produced at Kemp Bio Inc and the P2 viral stocks of PhiC31 integrase and NLS Cre Recombinase had titers of 1.7X10e8 and 1.2X10e8 pfu/ml, respectively.

Cell culture

HEK293 cells and HEK293 FolateR-floxSTOP cells were maintained in growth media consisting of DMEM + GlutaMAX, supplemented with 10% ES cell-qualified fetal bovine serum, and 1% nonessential amino acids.

Plasmid transfection

HEK293 cells (1×10^6) were resuspended in 1 ml of Resuspension Buffer provided with the Neon transfection kit, mixed gently and 120 ul of cells aliquoted into tubes containing DNA. The above Cell: DNA mix was collected in a 100 ul tip and electroporated at 1300V, 10ms, 3 Pulse in a Neon Electroporation System. Transfected cells were carefully transferred to 6 well dishes containing 3 mls of pre-warmed HEK293 media and media refreshed after 24 h.

BacMam transduction

HEK293 FolateR-floxSTOP cells were transduced with BacMam virus by removal of spent media and replacement with fresh media containing 5% v/v NLS Cre recombinase BacMam. Parallel wells were transfected with 5ug NLS Cre Recombinase vector using Lipofectamine 2000. The NLS-Cre expression cassette was kindly provided by William Stanford, Ottawa Hospital Research Institute. For examination of PhiC31 integrase, HEK293 cells transfected with GFP expression vector alone.

Parallel well contained cells that was co-transfected with PhiC31 Integrase vector via electroporation via Neon. Media from these transfected cells were replaced while conditions with only the GFP vector transfected into the cells were further incubated with varying concentration of BacMam virus ranging from 10-30% v/v. This range was chosen to off-set the control condition where the PhiC31 integrase was co-transfected with the cells 24 h earlier. Following overnight incubation of the cells with virus, media was replaced and 48 h post viral transduction; cells were cultured in media containing 200 ug/ml Hygromycin for 2 weeks.

Staining and flow cytometry

For live staining, cells were washed 1X with DMEM/F-12 and incubated with Anti-Folate Antibody Receptor conjugated with APC 1:100 dilution (in DMEM/F-12) for 60 min at 37°C. Cells were harvested for flow cytometry with 0.25% Trypsin EDTA. Data was collected using a BD FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Results and Discussion

For proof of concept of BacMam NLS-Cre, a stable HEK 293 cell line was previously engineered with the ability to activate a folate receptor gene following exogenous expression of Cre recombinase. Upon activation, expression of the folate receptor gene can be detected

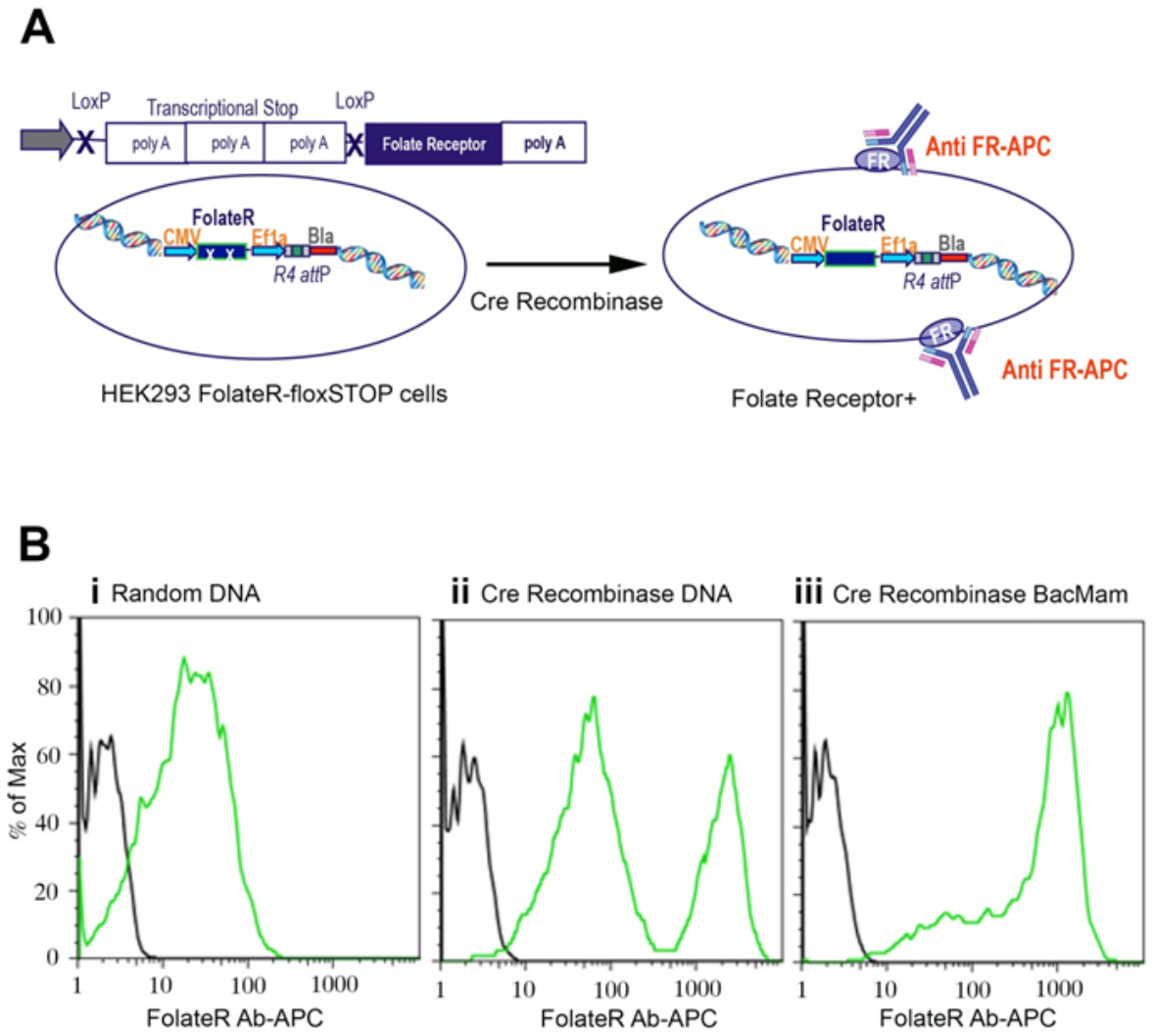

Figure 2: Functional NLS-Cre mediated Folate receptor activation. (A) HEK293 Folate R-flox STOP cell line was created by inserting a LoxP floxed Transcriptional stop in between the promoter and folate receptor expression cassette to disrupt expression. Removal of the floxed transcriptional stop via Cre Recombinase results in intact Receptor cassette enabling expression and cell surface presentation of the Folate Receptor that can be detected using antibodies. (B) Flow cytometry analysis of HEK293 FolateR-floxSTOP cells 48 h post transfection harvested and immunostained with Folate Receptor antibody conjugated to APC (Black – Unstained controls, Green – cells stained with Folate R Antibody conjugated to APC.). Cells were treated with either (i) Random DNA, (ii) Cre Recombinase expression DNA construct and (iii) BacMam virus expressing Cre recombinase.

using Anti Folate Receptor (Anti-FR) APC conjugated antibodies. Using Multisite Gateway, the expression cassette was designed such that the constitutive Cytomegalovirus (CMV) promoter driving exogenous folate receptor expression was disrupted by three poly adenylation sequences flanked with Lox P sites (Unpublished data). The expression of Cre recombinase allows for excision of the poly adenylation repeats, allowing the CMV promoter to drive folate receptor expression (Figure 2A). In all conditions below, the cells were first transfected, and 48 h post transfection, cells were harvested, stained using the Anti-FR APC antibody and analyzed by Flow cytometry (Figure 2B). A random DNA construct without Cre Recombinase was transfected to detect any background antibody staining (Figure 2B, i). In this control condition, one broad peak a with peak intensity at 20 represents the nonspecific-staining signal. "Functional excision was generated by transfection with

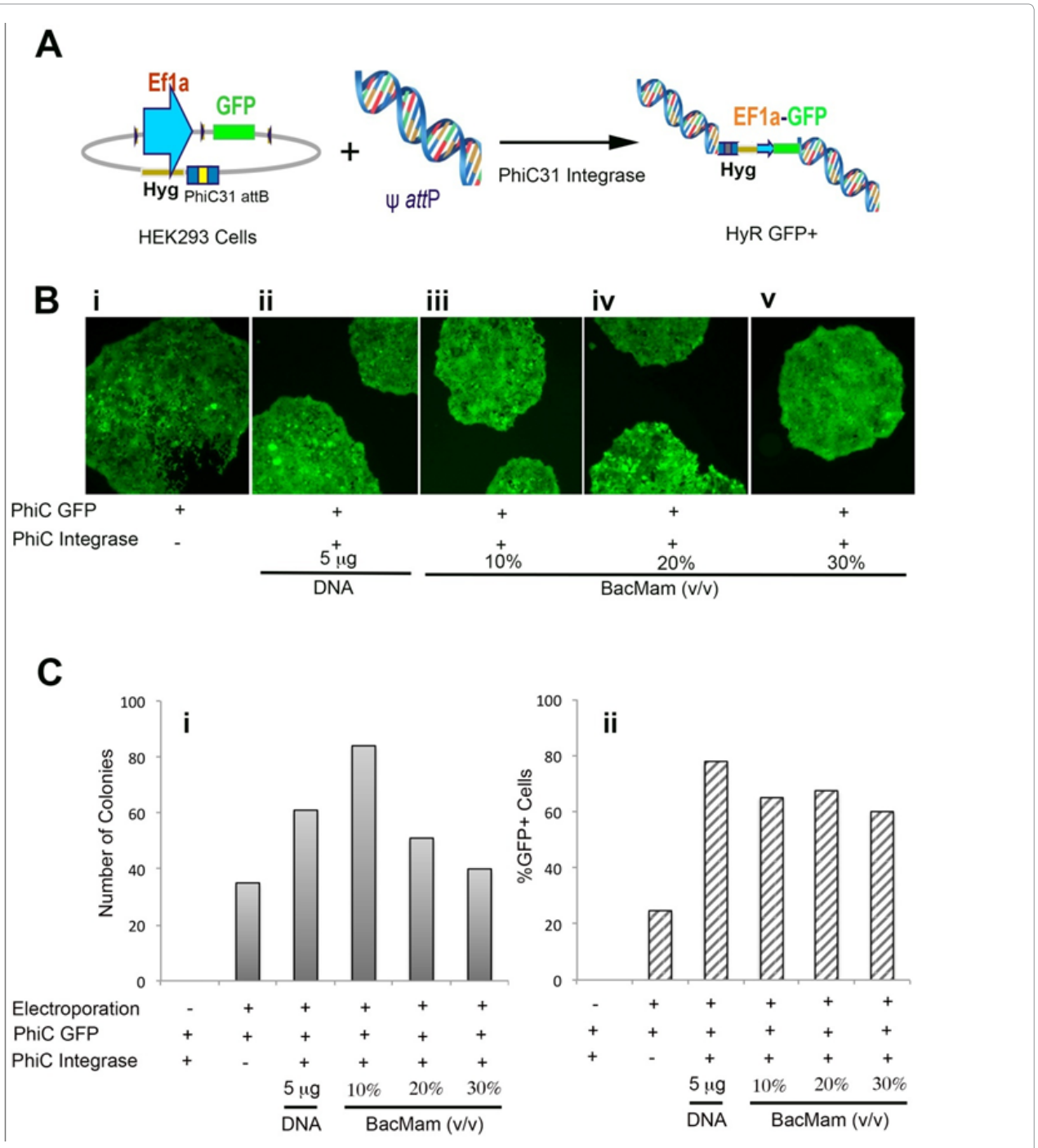

Figure 3: PhiC31 Integrase delivery via BacMam (A) Multisite Gateway Technology was used to create vectors with a backbone comprised of a Hygromycin selection cassette and an attB site for integration into a pseudo attP site in the mammalian genome, carrying an EF1a mammalian promoter driven GFP expression cassette. (B) Various combination of the GFP expression vector and absence and presence of PhiC31 integrase supplied either as DNA or BacMam virus resulted in HYG resistant GFP positive colonies. (C) To determine the efficiency of PhiC31 catalyzed genomic integration of the GFP expression construct, (i) Colony number was determined and, (ii) the clones pooled and analyzed by flow cytometry to determine % GFP + cells.

plasmid DNA driving Cre Recombinase expression. When the NLS-Cre plasmid was transfected into the cell line, the results show a second peak with increased intensity (>1000) of APC detection (Figure 2B,ii). This second peak represents cells that express Folate receptor resulting from excision of the transcriptional stop sequence by Cre recombinase. The presence of two peaks suggests a heterogeneous mixture of parental Flox STOP cells and Cre recombined Folate Receptor positive cells. This could be due to either incomplete action of the Cre Recombinase or inefficient delivery of DNA resulting in complete catalysis of floxSTOP excision only in a fraction of the cells. In contrast, cells with Cre recombinase delivered via BacMam showed a single prominent peak corresponding to cells that express folate receptor (Figure 2B,iii).

These results demonstrate that Cre recombinase delivery via BacMam displays greater efficacy of excision throughout the cell population compared to DNA delivery. Integration of an expression cassette via PhiC31 integrase has been reported in several cell types using vectors carrying an attB site that, in the presence of PhiC31 integrase, catalyzes the site-specific insertion of the vector in to various psuedo attP hot spots within the mammalian genome via sequence homology (Figure 3A). In addition, the presence of Hygromycin antibiotic cassette on the vectors enables drug selection of positive clones.

In this study we first show the integration of a GFP expression plasmid driven by Ef1alpha using the vector alone. Thirty-five colonies were generated post selection with Hygromycin. The resulting colonies are largely randomly integrated. Next, PhiC31 Integrase was co-transfected with the GFP expression vector. As expected, there was an increase in the number of GFP positive colonies with a total of sixty-one GFP+ HygR colonies. In order to normalize against an optimized DNA concentration, various volumes of virus ranging from 10-30 v/v to the total culture media was used for transduction and expression of the integrase in tandem with lipid transfection of the GFP expression vector. All conditions resulted in GFP+ HygR colonies albeit with varying efficiencies and uniformity of GFP expression (Figure 3B). To further dissect the efficiency of stable clone generation and homogeneity of expression, the total number of clones obtained under each condition was counted and the clonal population pooled to assess the percent of GFP positive cells (Figure 3C). The total number of clones was highest for cells electroporated with the PhiC GFP and PhiC31 integrase delivered via BacMam at 10%v/v (Figure 3C, i). Higher concentrations of PhiC31 integrase BacMam at 20 and 30% v/v resulted in lesser number of cells, that could likely be due to toxicity of the higher concentration of the virus on transfected cells. Although the number of colonies obtained between 5 ug DNA and 10% v/v BacMam cannot be directly correlated since the amounts cannot be directly compared and the timing of addition is different, the higher number of colonies obtained with BacMam suggested that delivery of PhiC31 via BacMam is a viable approach.

To further examine if all the HygR colonies were also GFP+, clones obtained under each condition were pooled and the cells harvested for flow cytometry analysis (Figure 3C,ii). Interestingly the number of GFP+ cells was comparable between all samples treated with PhiC31 integrase irrespective of whether it was delivered via DNA or BacMam at varying concentrations. In contrast, cells transfected with PhiC31 attB containing GFP vector alone had a significantly lower percentage of GFP+ cells. This observation is consistent with Figure 3B, which shows that HygR colonies obtained in the presence of PhiC31 integrase have a more uniform GFP expression compared to clones generated in its absence. The low GFP expression in the absence of PhiC31 integrase could be either due to breakage of the vector in the GFP expression cassette prior to genomic integration or random insertion at genomic sites that fail to support robust expression necessary for visualization of fluorescent proteins. As supported by several published reports [5,9,10], the presence of PhiC31 integrase directs integration into sites in the mammalian genome that are less prone to silencing, thus supporting higher expression.

This report demonstrates the ease and utility of delivery catalyzing enzymes via BacMam virus. The advantages of this mode of delivery are safety of using a non-integrating system, ease of use and efficiency of BacMam transduction in hard-to-transfect cells.

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