Chapter 2

Mutagenesis and Genome Engineering of Epstein–Barr Virus in Cultured Human Cells by CRISPR/Cas9

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Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 nuclease (Cas9) system is a powerful genome-editing tool for both chromosomal and extrachromosomal DNA. DNA viruses such as Epstein–Barr virus (EBV), which undergoes episomal replication in human cells, can be effectively edited by CRISPR/Cas9. We have demonstrated targeted editing of the EBV genome by CRISPR/Cas9 in several lines of EBV-infected cells. CRISPR/Cas9-based mutagenesis and genome engineering of EBV provides a new method for genetic analysis, which has some advantages over bacterial artificial chromosome-based recombineering. This approach might also prove useful in the cure of EBV infection. In this chapter, we use the knockout of the BART promoter as an example to detail the experimental procedures for construction of recombinant EBV in human cells.

Key words RNA-guided genome editing, Episomal viral DNA genome, Epstein–Barr virus, Genetic analysis of Epstein–Barr virus, Cure of Epstein–Barr virus infection

Abbreviations

BAC	Bacterial artificial chromosome
Cas9	CRISPR associated protein 9 nuclease
CRISPR	Clustered regularly interspaced short palindromic repeats
DSB	Double-strand break
EBV	Epstein–Barr virus
GFP	Green fluorescent protein
gRNA	Guide RNA
MOI	Multiplicity of infection
PAM	Protospacer adjacent motif
pBART	*Bam*HI-A region rightward transcript promoter
pCMV	Cytomegalovirus promoter
PCR	Polymerase chain reaction

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1 Introduction

Genetic studies are important to all areas of biology. In virology, targeted mutation of particular genetic elements on the viral genome helps to understand their function. In the early years, genomes of herpesviruses were engineered using homologous recombination in eukaryotic cells [1–4]. Subsequently, the successful cloning of herpesviral genomes into bacterial artificial chromosomes (BACs) greatly facilitated viral genome manipulation in prokaryotic cells [5–8]. However, in γ-herpesviruses including EBV and Kaposi sarcoma-associated herpesvirus, the titers of viruses recovered from BAC-transfected producer cell lines are usually very low for unknown reasons, and some transfectants even lose their ability to support lytic viral replication [7]. The low efficiency in generating high-quality γ-herpesvirus producing cell lines has become the bottleneck issue in the use of EBV BAC in the field [7]. Intensive screening of stable cells is required to obtain high-quality EBV producing cell lines and this hinders the application of EBV BAC.

In order to provide an alternative method for mutagenesis and genome engineering of EBV, we harnessed the emerging CRISPR/Cas9 technology for targeted editing of the EBV genome. CRISPR/Cas9 was originally discovered as part of the adaptive immune system in bacteria but has now been developed into a powerful method for double-strand break (DSB)-induced genome editing in eukaryotic cells [9, 10]. The EBV genome exists in infected cells as multicopy episomes [11] and this poses a unique challenge for CRISPR/Cas9 editing. Whether the multicopy EBV episome could be efficiently cleaved by CRISPR/Cas9 is one major concern and how the correctly edited version of the EBV genome might be separated from predominantly unedited viral DNA is another critical issue. In this regard, we have not only demonstrated the feasibility of CRISPR/Cas9 editing of the EBV genome but have also designed and tested different approaches to isolate the correctly edited recombinant EBV [12].

In this chapter, we detail how we harnessed CRISPR/Cas9 to edit the EBV genome in human cells. First, we describe the steps and criteria for optimal guide RNA (gRNA) design for EBV editing (*see* Subheading 3.1). Second, we provide the step-by-step procedures for CRISPR/Cas9 editing of the EBV genome in mammalian cells (*see* Subheading 3.2). The cell lines harboring edited EBV are helpful for genetic study of EBV. Third, we describe the procedures for the isolation of pure and infectious recombinant EBV created by CRISPR/Cas9 (*see* Subheading 3.3). The recombinant EBV can be produced and used for further infection experiments. Particularly, we supplied the protocol for insertion of the DsRed fluorescent marker to facilitate the recovery of mutant EBV. The methods described are generally applicable to the creation of both gene disruption and gene replacement in not only EBV but also other DNA viruses.

2 Materials

2.1 Molecular Biology Materials

1. PX459 vector (kindly provided by Dr. Feng Zhang, MIT, Cambridge MA).
2. Genomic DNA Purification Kit (e.g., Wizard, Promega).
3. GeneJuice (Novagen).
4. TransIT-Keratinocyte Transfection Reagent (Mirus).
5. Puromycin.
6. Filter papers and punches.
7. Agarose.
8. Agarose gel electrophoresis system.
9. RT-PCR reagents.
10. Western blotting reagents and apparatus.
11. Commercial RPMI 1640 medium (Life Technologies).
12. Goat-anti-human IgG.
13. 1× PBS.
14. pDsRed2-C1 vector (Clontech).
15. BZLF1 and gp110 expression plasmids.
16. Flow cytometer with sorting capability (e.g., BD FACSAria SORP).
17. Hi-Fidelity DNA polymerase.
18. *Dpn*I restriction enzyme.
19. Commercial DNA gel and PCR cleanup kit.
20. 1.20 μm syringe filter.
21. Amicon® Ultra-15 Centrifugal filter Ultracel®-100 kDa cutoff.

3 Methods

3.1 gRNA Design and Cloning

The steps and criteria for optimal gRNA design are described based on using the deletion of the BART promoter in the EBV genome as an example. In our design, two gRNAs (pB1 and pB2) are employed to flank the whole BART promoter (pBART) region for deletion (*see* **Note 1**). The pSpCas9(BB)-2A-Puro (PX459)-based CRISPR/Cas9 system [13], kindly provided by Dr. Feng Zhang, is accessed through Addgene (https://www.addgene.org/62988/), and is used in the following protocol.

1. The target region in the EBV genome is searched for a 19-bp sequence followed by the protospacer adjacent motif (PAM) NGG at the 3′ end. Two gRNAs flanking the target region are

designed (*see* **Note 2**). For optimal gRNA binding, the GC content of the 19-bp target sequence is preferably within 40–60%. The sequences of the pBART-gRNAs are as follows:

gRNA-pB1, TAATTGCAGTGGACCCCGG AGG^{PAM}
gRNA-pB2, AAGAAGCTCCTCAGCAACA TGG^{PAM}

2. The target sequence is subjected to off-target analysis. Human and EBV genome sequences in the National Center for Biotechnology Information nucleotide databases are BLAST searched for matches to the 19-bp sequence together with NGG (e.g., TAATTGCAGTGGACCCCGGNGG for gRNA-B1). The stringency of the off-target analysis could be adjusted according to the purpose of the study. Only gRNAs with limited off-target hits are chosen. In our case, a sequence with a perfect match to PAM together with a match of >10 bp in the 19-bp region is avoided.
3. The 20-bp gRNA sequence with the first nucleotide being G is inserted into the PX459 vector. The addition of a G is required for optimal expression from the U6 promoter. The PX459 vector contains both gRNA and Cas9 expression cassettes.
4. The gRNA insert can be made by annealing the sense and antisense oligonucleotides. The annealed oligonucleotides are then inserted into the PX459 vector using the Zhang Lab General Cloning Protocol at the following website: (http://www.addgene.org/crispr/zhang/).

3.2 EBV Genome Editing

We have used several EBV-infected epithelial cell lines for CRISPR/Cas9 editing of the EBV genome [12]. These include the EBV-infected human embryonic kidney cell line HEK293-EBV, the human nasopharyngeal carcinoma cell lines HK1-EBV and C666-1, the human gastric adenocarcinoma cell line AGS-BX1 as well as the EBV-infected human telomerase reverse transcriptase-immortalized normal nasopharyngeal epithelial cell line NP460-EBV. CRISPR/Cas9 editing of the EBV genome is performed directly inside these cells. Cells containing the edited virus are recovered by puromycin selection. The protocol should generally be amenable to all other EBV-infected cell lines that are not too difficult to transfect, including some B lymphocytic lines.

1. Grow approximately 2×10^5 EBV-infected epithelial cells in a 6-well tissue culture plate.
2. After 24 h, transfect the cells with 1 μg of gRNA1 and 1 μg of gRNA2 with GeneJuice and TransIT transfection reagents (*see* **Note 3**).
3. Harvest the cells 72 h post-transfection. Half of the cells are collected for genomic DNA extraction. Genomic DNA is extracted using a genomic DNA purification kit.

4. Perform PCR using primers flanking the deleted region to screen for the desired CRISPR/Cas9-edited mutant virus. Successful editing will result in an additional amplicon of smaller size.
5. Transfer the remaining half of the cells into a 10-cm tissue culture dish. Twenty-four hours later, puromycin is added to select for the stable cells which contain the mutant virus (*see* **Note 4**).
6. Replace the drug-containing medium with fresh medium and cells after 48 h after puromycin treatment. Allow the cells to grow for 1 more week.
7. After 1 week, visible colonies of the selected cells should appear on the plate. Single colonies are picked by using filter paper (*see* **Note 5**).
8. Place a pre-soaked filter paper (with trypsin–EDTA) on top of the single cell colony at 37 °C for 3 min. The filter paper is then transferred with a pair of sterile forceps onto a 6-well tissue culture plate containing 2 ml of fresh culture medium. Shake the filter paper in the medium to make sure that the attached cells on the filter paper are detached into the well. The detached cells are allowed to recover and grow for 4 more days.
9. Verify the surviving clones by PCR as described in **steps 3** and **4** above. PCR products derived from both edited and unedited EBV genomes are analyzed by agarose gel electrophoresis. In general, most of the surviving clones contain a mixture of wild-type and edited forms of EBV (*see* **Note 6**). In our pBART knockout experiment conducted in HEK293-EBV cells, 3 out of 50 clones were shown to be deprived of the unedited form of EBV carrying the pBART-deleted mutant virus only [12].
10. Perform RT-PCR and Western blotting to verify the disruption of the target gene product in the puromycin-selected cell clones.

3.3 Recombinant EBV

1. To generate an EBV-infected cell line that meets all the special requirements for the creation of a recombinant EBV by CRISPR/Cas9 editing, we have established HEK293-BX1 cells by coculturing HEK293 with Akata-BX1 cells. HEK293 cells are commonly used as EBV producer cells in BAC recombineering [6, 7]. They are highly susceptible to transfection and induction of lytic replication. Establishment of HEK293-BX1 cells for the conduction of CRISPR/Cas9 editing can therefore facilitate the construction, isolation, and production of recombinant EBV. On the other hand, insertion of a selectable marker or fluorescent reporter into the EBV genome can enable drug selection and mutant tracing, which are desirable for the isolation of recombinant EBV. To this end, we provide a protocol for the addition of DsRed fluorescent marker into EBV genome during CRISPR/Cas9 editing. This greatly facilitates tracing and recovery of mutant viruses. Additionally, we also describe

an experimental approach to establish HEK293 producer cells of the mutant EBV through reinfection and sorting of DsRed$^+$ single cells. This allows rapid and efficient isolation of the desired mutant virus created by CRISPR/Cas9 from a mixture of unedited and edited EBV genomes.

3.4 HEK293-BX1 Cells

1. Treat approximately 1×10^5 Akata-BX1 cells with 0.5% of goat-anti-human IgG in a 20-mm tissue culture dish for induction of lytic replication.
2. Wash the Akata-BX1 cells after 24 h with 1× PBS and collect by centrifugation at $200 \times g$ for 5 min.
3. Resuspend the cell pellets in 2 ml RPMI 1640. The IgG-induced Akata-BX1 cells serve as the source of EBV for the infection of HEK293 cells.
4. Grow approximately 1×10^5 HEK293 cells in a 6-well tissue culture plate 1 day before infection.
5. Wash the HEK293 cells with 1× PBS and combine with the IgG-induced Akata-BX1 cells.
6. After coculturing for 72 h, wash away the Akata-BX1 cells from the HEK293 cells with 1× PBS.
7. Observe the infection of HEK293 cells under a fluorescent microscope. Successfully infected HEK293-BX1 cells are green fluorescent protein (GFP)-positive because the BX1 virus carries a GFP fluorescent marker [12]. The efficiency of EBV infection through coculturing is usually in the range of 5–10%.
8. Trypsinize the cells and transfer them into a 10-cm tissue culture dish. Cells are then grown to confluence.
9. Single-cell sort GFP-positive cells onto a 96-well tissue culture plate by BD FACSAria SORP.
10. Recover the single cell-sorted HEK293-BX1 cells in a 96-well tissue culture plate over the next 7–10 days.

3.5 DsRed Insertion

1. The pCMV-DsRed fragment containing the cytomegalovirus promoter (pCMV) was PCR-amplified from plasmid pDsRed2-C1 using KAPA HiFi DNA polymerase. Primers with an EBV homology arm of 50 bp are used for the amplification. For the knockout of pBART, the primers are 5′-ATGGTATTG GTCGTCTTCTTCCCCTGCAGAGTAATTGCA GTGGACCCCGGTGTTCTTTCCTGCGTTATCCC-3′ and 5′-TAGTGCCTACGTGACTCCCTGCTCCTGCCAGTT TCCCTTCGAGGTCTCCATAAGGGATTTTGC-3′. The amplification primers for pCMV-DsRed are underlined and the EBV homology arms are located at the 5′ end.
2. Treat the pCMV-DsRed fragment with 1 μl of *Dpn*I at 37 °C for 60 min to remove the pDsRed2-C1 template.

3. The pCMV-DsRed fragment is gel-purified by using a high-quality gel and PCR clean up kit.
4. One day before transfection, approximately 2×10^5 of HEK293-BX1 cells are grown in 6-well tissue culture plate.
5. Transfect cells with 0.2 μg of gRNA1, 0.2 μg of gRNA2 and 1.6 μg of pCMV-DsRed fragment.
6. Collect half of the cells 72 h post-transfection for PCR verification. Insertion of DsRed into the target region is confirmed by using primers complementary to the junction between the target region and the DsRed cassette.

3.6 HEK293 Producer Cells

1. Transfer the remaining half of the cells into 4 × 10-cm tissue culture dishes.
2. Next day, transfect the cells in the culture dish with 4 μg each of BZLF1 and gp110 expression plasmids to induce EBV lytic replication (*see* **Note 7**).
3. After 24 h, replace the culture medium of the transfected cells with fresh RPMI 1640. The transfected cells are grown for 5 more days for recombinant EBV production.
4. Harvest the supernatants 6 days after transfection. All supernatants are filtered through a 1.20-μm syringe filter before infection.
5. Concentrate 40 ml of supernatant into 1 ml by using an Amicon® Ultra-15 Centrifugal filter Ultracel®-100 kDa. The Centricon is spun at 1400 × *g* for concentrating. It usually takes somewhere in the range of 30–45 min to concentrate 40 ml of supernatant to 1 ml. The concentrated virus can be stored at 4 °C for 1 week.
6. Grow approximately 2×10^5 HEK293 cells in a 6-well tissue culture plate 1 day before infection.
7. Add 1 ml of concentrated virus to 2×10^5 of HEK293 cells. The culture medium is replaced with fresh RPMI 1640 after 24 h.
8. Seventy-two hours after infection, monitor cells under a fluorescent microscope. Cells infected with wild-type EBV are GFP-positive, whereas cells infected with recombinant EBV are doubly positive for GFP and DsRed (*see* **Note 8**).
9. Sort single cells doubly positive for GFP and DsRed onto a 96-well tissue culture plate by BD FACSAria SORP.
10. Recover the sorted HEK293-ΔEBV-DsRed single cells in a 96-well tissue culture plate over the course of 7–10 days.
11. Test the recovered cells for recombinant virus production. Usually the producer cells yield 1×10^5 to 1×10^6/ml green Raji units of recombinant virus.

12. Verify the genomic pattern of the recombinant virus by PCR and deep sequencing.
13. Once verified, the recombinant viruses are now ready for use in other infection assays.

4 Notes

1. Compared to the single gRNA approach, using two gRNAs to splice out the target region enhances the editing efficiency and facilitates the subsequent screening process.
2. For abrogation of protein expression, the start codon and the first exon should preferably be removed. For other regulatory elements, the complete target region can be deleted by two gRNAs.
3. HEK293-EBV, HK1-EBV and AGS-BX1 cells are transfected with GeneJuice (Novagen) in the ratio of 1 μg of DNA to 3 μl of GeneJuice. C666-1 and NP460-EBV are transfected with TransIT-Keratinocyte Transfection Reagent (Mirus) in the ratio of 1 μg of DNA to 3 μl of TransIT Reagent.
4. PX459 vector contains a puromycin selection marker. For HEK293-EBV cells, 3 μg/ml of puromycin is used. For NP460-EBV and AGS-BX1 cells, 2 μg/ml of puromycin is used. For C666-1 and HK1-EBV cells, 0.5 μg/ml of puromycin is used.
5. Filter papers are prepared by punches. The punched filter paper is autoclaved before use.
6. EBV maintains 5–100 copies of covalently closed circular genomic DNA in latently infected cells [11]. Inside a single cell, some copies of the EBV genome may escape from CRISPR/Cas9 editing. Extensive screening is required to obtain stable cells with the pure EBV mutant.
7. BZLF1 is the key transcriptional activator mediating the switch between latent and lytic replication of EBV. gp110 is the viral glycoprotein which remarkably enhances the ability of EBV to infect human cells [7].
8. HEK293 cells are infected with a low multiplicity of infection (MOI) of EBV. Under low MOI conditions, most of the HEK293 cells will be infected with one particle of EBV, either a wild type or a recombinant.

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