Mutagenesis and genome engineering of Epstein-Barr virus in cultured human cells by CRISPR/Cas9

Kit-San Yuen[1], Chi-Ping Chan[1], Kin-Hang Kok[2], Dong-Yan Jin[1,*]

[1]School of Biomedical Sciences, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong

[2]Department of Microbiology, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong

*: Corresponding author

E-mails: yuenkitsan0119@gmail.com; khkok@hku.hk; chancp10@hku.hk; dyjin@hku.hk

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 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 used the knockout of 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	BamHI-A region rightward transcript promoter
pCMV	Cytomegalovirus promoter
PCR	Polymerase chain reaction

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 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 EBV genome. CRISPR/Cas9 is originally 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). 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, 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 EBV genome but have also designed and tested different approaches to isolate the correctly edited recombinant EBV (12).

In this chapter we detailed how we harnessed CRISPR/Cas9 to edit EBV genome in human cells. First, we described the steps and criteria for optimal guide RNA (gRNA) design for EBV editing (Subheading 3.1). Second, we provided the step-by-step procedures for CRISPR/Cas9 editing of EBV genome in mammalian cells (Subheading 3.2). The cell lines harboring edited EBV are helpful for genetic study of EBV. Third, we documented the procedures for the isolation of pure and infectious recombinant EBV created by CRISPR/Cas9 (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

1. PX459 vector (kindly provided by Dr. Feng Zhang, Massachusetts Institute of Technology, USA)
2. Wizard® Genomic DNA Purification Kit (Promega)
3. Puromycin (Sigma)
4. Filter papers and punches
5. RPMI 1640 medium (Life Technologies)
6. Goat-anti-human IgG (Millipore)
7. 1× PBS
8. pDsRed2-C1 vector (Clontech)
9. KAPA HiFi DNA polymerase (Kapabiosystems)
10. DpnI restriction enzyme (New England Biolabs)
11. Wizard® SV Gel and PCR Clean-Up System (Promega)
12. 1.20 µm syringe filter (Satorius Stedim)
13. Amicon® Ultra-15 Centrifugal filter Ultracel®-100K (Millipore, 100000 NMWL)

3. Methods

3.1 gRNA design and cloning

The steps and criteria for optimal gRNA design were described 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, Massachusetts Institute of Technology, USA through Addgene (https://www.addgene.org/62988/) is used in the following protocol.

1. Target region in the EBV genome is searched for 19bp 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 19bp target sequence is preferable to be 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 19bp 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 sequence with perfect match to PAM together with a match of >10bp in the 19bp region is avoided.

3. The 20bp gRNA sequence with the first nucleotide being G is inserted into PX459 vector. The addition of G is required for optimal expression from the U6 promoter. 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 Zhang Lab General Cloning Protocol (http://www.addgene.org/crispr/zhang/).

3.2 CRISPR/Cas9 editing of EBV genome in mammalian cells

We have used several EBV-infected epithelial cell lines for CRISPR/Cas9 editing of EBV genome (12). These include EBV-infected human embryonic kidney cell line HEK293-EBV, human nasopharyngeal carcinoma cell lines HK1-EBV and C666-1, human gastric

adenocarcinoma cell line AGS-BX1 as well as EBV-infected human telomerase reverse transcriptase-immortalized normal nasopharyngeal epithelial cell line NP460-EBV. CRISPR/Cas9 editing of 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. Approximately 2×10^5 of EBV-infected epithelial cells are grown in 6-well tissue culture plate.
2. After 24 hours, cells are transfected with 1 μg of gRNA1 and 1 μg of gRNA2 (See Note 3).
3. Cells are harvested 72 hours post-transfection. Half of the cells are collected for genomic DNA extraction. Genomic DNA is extracted using the Wizard® Genomic DNA Purification Kit (Promega).
4. PCR primers flanking the deleted region are used to screen for the desired CRISPR/Cas9-edited mutant virus. Successful editing will result in an additional amplicon of smaller size.
5. The remaining half of the cells are transferred onto 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. At 48 hours after treatment with puromycin, the drug-containing medium is replaced by fresh medium and cells are allowed to grow for one more week.
7. After 1 week, visible colonies of the selected cells appear on the plate. Single colonies are picked by using filter paper (See Note 5).
8. A filter paper is pre-soaked with trypsin/EDTA and placed on top of the cell colony at 37°C for 3 minutes. 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. The survived cell clones are verified by PCR as in Steps 3 and 4. PCR products derived from both edited and unedited EBV genome are analyzed by agarose gel electrophoresis. In general, most of the survived 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. RT-PCR and Western blotting are performed to verify the disruption of the

target gene product in the puromycin-selected cell clones.

3.3 Isolation of recombinant EBV created by CRISPR/Cas9 editing

To generate an EBV-infected cell line that meets all special requirements for the creation of recombinant EBV by CRISPR/Cas9 editing, we have established HEK293-BX1 cells through co-culture of 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 EBV genome can enable drug selection and mutant tracing, which are desirable in the isolation of recombinant EBV. To this end, we provided 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 described an experimental approach to establish HEK293 producer cells of the mutant EBV through re-infection 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.

3.3.1 Establishment of HEK293-BX1 cells

1. Approximately 1×10^5 of Akata-BX1 cells are treated with 0.5% of goat-anti-human IgG (Millipore) in 20-mm tissue culture dish for induction of lytic replication.
2. After 24 hours, Akata-BX1 cells are washed with 1× PBS and collected by centrifugation at 200 × g for 5 minutes.
3. The cell pellets are resuspended in 2 ml RPMI 1640. The IgG-induced Akata-BX1 cells serve as the source of EBV for the infection of HEK293 cells.
4. Approximately 1×10^5 of HEK293 cells are grown in 6-well tissue culture plate one day before infection.
5. HEK293 cells are washed with 1× PBS and added with IgG-induced Akata-BX1 cells.
6. After co-culturing for 72 hours, Akata-BX1 cells are washed away from HEK293 cells with 1× PBS.
7. Infection of HEK293 cells are observed under 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 co-culturing is usually 5-10%.

8. Cells are trypsinized and transferred onto a 10-cm tissue culture dish. Cells are grown till confluence.
9. GFP-positive cells are then single cell-sorted onto 96-well tissue culture plate by BD FACSAria SORP (BD Biosciences).
10. The single cell-sorted HEK293-BX1 cells are recovered in 96-well tissue culture plate for 7-10 days.

3.3.2 Insertion of DsRed marker into EBV genome

1. pCMV-DsRed fragment containing the cytomegalovirus promoter (pCMV) was PCR-amplified from plasmid pDsRed2-C1 (Clontech) using KAPA HiFi DNA polymerase (Kapabiosystems). Primers with an EBV homology arm of 50 bp are used for the amplification. For the knockout of pBART, the primers are 5'-ATGGTATTGGTCGTCTTCTTCCCCTGCAGAGTAATTGCAGTGGACCCCGGTGTTCTTTCCTGCGTTATCCC-3' and 5'-TAGTGCCTACGTGACTCCCTGCTCCTGCCAGTTTCCCTTCGAGGTCTCCATAAGGGATTTTGC-3'. The amplification primers for pCMV-DsRed are underlined and the EBV homology arms are located at the 5' end.
2. The pCMV-DsRed fragment is then treated with 1 µl of DpnI (New England Biolabs) at 37°C for 60 minutes to remove the pDsRed2-C1 template.

3. The pCMV-DsRed fragment is gel-purified by using Wizard® SV Gel and PCR Clean-Up System (Promega).
4. One day before transfection, approximately 2×10^5 of HEK293-BX1 cells are grown in 6-well tissue culture plate.
5. Cells are then transfected with 0.2 μg of gRNA1, 0.2 μg of gRNA2 and 1.6 μg of pCMV-DsRed fragment.
6. Half of the cells are collected 72 hours post-transfection for PCR verification. Insertion of DsRed into the target region are confirmed by using primers complementary to the junction between the target region and the DsRed cassette.

3.3.3 Establishment of HEK293 producer cells for recombinant EBV

1. The remaining half of the cells are transferred onto 4× 10-cm tissue culture dishes.
2. In the next day, cells in the dish are transfected with 4 μg each of BZLF1 and gp110 expression plasmids to induce EBV lytic replication (See Note 7).
3. After 24 hours, culture medium of the transfected cells is replaced by fresh RPMI 1640. The transfected cells are grown for 5 more days for recombinant EBV production.

4. The supernatants are harvested 6 days after transfection. All supernatants are filtered through 1.20-μm syringe filter before infection.
5. Forty milliliters of supernatant are concentrated into 1 ml by using Amicon® Ultra-15 Centrifugal filter Ultracel®-100K (Millipore, 100000 NMWL). The Centricon is spun at 1400 × g for concentration. It usually takes 30-45 minutes to concentrate 40 ml of supernatant. The concentrated virus can be stored at 4°C for one week.
6. Approximately 2×10^5 of HEK293 cells are grown in 6-well tissue culture plate one day before infection.
7. One milliliter of concentrated virus is added to 2×10^5 of HEK293 cells. The culture medium is replaced with fresh RPMI 1640 after 24 hours.
8. Seventy-two hours after infection, HEK293 cells infected with $DsRed^+$ recombinant EBV are monitored under 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. Cells doubly positive for GFP and DsRed are then single cell-sorted onto 96-well tissue culture plate by BD FACSAria SORP (BD Biosciences).
10. The single cell-sorted HEK293-ΔEBV-DsRed cells are recovered to 96-well tissue culture plate for 7-10 days.

11. The recovered cells are then tested for recombinant virus production. Usually the producer cells yield 1×10^5 -1×10^6 ml^{-1} green Raji units of recombinant virus.
12. The genomic pattern of the recombinant virus will also be verified by PCR and deep sequencing.
13. The recombinant viruses are ready for use in other infection assays.

4. Notes

1. Compared to 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 NP460EBV 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 puromycin selection marker. For HEK293-EBV cells, 3

μg/ml of puromycin is used. For NP460EBV 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 genome 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 low multiplicity of infection (MOI) of EBV. Under low MOI, most of the HEK293 cells will be infected with one particle of EBV, either wild-type or recombinant.

Acknowledgements

This work was supported by Hong Kong Health and Medical Research Fund (11100602 and 12110962), S.K. Yee Medical Research Fund (2011) and Hong Kong Research Grants Council (AoE/M-06/08, HKU1/CRF/11G, C7011-15R and T11-707/15-R).

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