CRISPR/Cas9-mediated genome editing of Epstein–Barr virus in human cells

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The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated 9) system is a highly efficient and powerful tool for RNA-guided editing of the cellular genome. Whether CRISPR/Cas9 can also cleave the genome of DNA viruses such as Epstein–Barr virus (EBV), which undergo episomal replication in human cells, remains to be established. Here, we reported on CRISPR/Cas9-mediated editing of the EBV genome in human cells. Two guide RNAs (gRNAs) were used to direct a targeted deletion of 558 bp in the promoter region of BART (BamHI A rightward transcript) which encodes viral microRNAs (miRNAs). Targeted editing was achieved in several human epithelial cell lines latently infected with EBV, including nasopharyngeal carcinoma C666-1 cells. CRISPR/Cas9-mediated editing of the EBV genome was efficient. A recombinant virus with the desired deletion was obtained after puromycin selection of cells expressing Cas9 and gRNAs. No off-target cleavage was found by deep sequencing. The loss of BART miRNA expression and activity was verified, supporting the BART promoter as the major promoter of BART RNA. Although CRISPR/Cas9-mediated editing of the multicopy episome of EBV in infected HEK293 cells was mostly incomplete, viruses could be recovered and introduced into other cells at low m.o.i. Recombinant viruses with an edited genome could be further isolated through single-cell sorting. Finally, a DsRed selectable marker was successfully introduced into the EBV genome during the course of CRISPR/Cas9-mediated editing. Taken together, our work provided not only the first genetic evidence that the BART promoter drives the expression of the BART transcript, but also a new and efficient method for targeted editing of EBV genome in human cells.

INTRODUCTION

As part of the adaptive immune response in bacteria and archaea, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) systems employ two small RNAs to direct the Cas nuclease to specifically degrade nucleic acids from invading viruses (Terns & Terns, 2014). RNA-guided genome editing based on one of these systems termed CRISPR/Cas9 has recently emerged as a powerful and versatile tool for targeted engineering of the cellular genome (Mali et al., 2013; Hsu et al., 2014). To facilitate the use of the CRISPR/Cas9 system for genome editing in mammalian cells, a new bicistronic vector that expresses both Cas9 nuclease and a functional guide RNA (gRNA), which sufficiently directs sequence-specific recognition, has been developed (Ran et al., 2013). The CRISPR/Cas9 system has been used successfully to generate knockout cells and animals (Mali et al., 2013; Hsu et al., 2014). Whether the CRISPR/Cas9 system can also be used to efficiently manipulate genomes of DNA viruses, which replicate extrachromosomally to high copy numbers, remains to be fully established. In this regard, one recent report indicated that the CRISPR/Cas9 system could indeed be used to introduce indel mutations in target genes with high efficiency in the genomes of adenovirus and herpes simplex virus type 1 (HSV-1) in freshly infected human cells in which the virus undergoes lytic replication (Bi et al., 2014). However, it remains to be seen whether CRISPR/Cas9 can also cleave the large genomes of other DNA viruses, particularly in persistently infected cells. In addition, methods to isolate pure clones of the edited virus for phenotypic characterization have not been described.

Epstein–Barr virus (EBV) establishes a lifelong persistent infection in 95% of all adults. Although it causes no disease in healthy carriers, EBV infection is aetiologically associated...
with different types of lymphoid and epithelial malignancies, such as Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma and gastric cancer, in a small subset of individuals (Raab-Traub, 2012). Although >100 viral proteins are expressed during productive replication of EBV, only a limited set of ≤14 viral transcripts are found during latent infection (Lieberman, 2013). To investigate how these transcripts expressed in latently infected cells might contribute to EBV-induced cellular transformation and oncogenesis, it is desirable to introduce specific mutations into the EBV genome. This was accomplished initially by homologous recombination in human cells, which in recent years has given way to recombining in bacterial artificial chromosomes (BACs) carrying the EBV genome (Feederle et al., 2010). Although BAC technology is highly efficient and powerful, there are technical hurdles and difficulties that limit its use in some circumstances. If the emerging CRISPR/Cas9 technology can be adopted for targeted editing of the EBV genome in human cells, it will add a new tool that might be complementary to BAC recombining in bacteria. EBV maintains between five and 100 copies of the covalently closed circular genome in latently infected cells (Adams & Lindahl, 1975). This might constitute a major technical challenge to CRISPR/Cas9-mediated editing. Thus, it will be of interest to determine whether the multicopy episomal genome of EBV in latently infected cells might be susceptible to CRISPR/Cas9-mediated editing. It will also be intriguing to see whether a selectable marker can be introduced into the recombinant virus during CRISPR/Cas9-mediated editing of EBV genome.

In this study, we set out to explore CRISPR/Cas9-mediated editing of the EBV genome in cultured human cells. Our aim was to construct a recombinant EBV that does not express BART (BamHI A rightward transcript) – a major viral transcript expressed in latently infected cells. BART RNA is transcribed from two RNA polymerase II-dependent promoters P1 and P2 (Chen et al., 2003, 2005b). It encodes two clusters of microRNAs (miRNAs) that target viral and cellular genes to promote cell survival, evade immune response, modulate viral gene expression and induce carcinogenesis (Umbach & Cullen, 2009; Lo et al., 2012; Riley et al., 2012). As the BART transcript is most abundantly expressed in latently infected epithelial cells (Cai et al., 2006; Choy et al., 2008b), we chose these cells as the target cells in our study. The strategy that we used to abrogate BART expression was to cleave out both P1 and P2 promoters by CRISPR/Cas9. By characterizing the resulting recombinant virus, we provided the first genetic evidence that P1 and P2 are indeed the major promoters for the transcription of BART RNA. We also established the feasibility of CRISPR/Cas9-mediated editing of the EBV genome in latently infected cells. We assessed the cleavage efficiency and provided two experimental approaches to obtain pure recombinant virus with the desired deletion in the BART promoter (pBART). We also successfully introduced a selectable marker into the EBV genome during the course of CRISPR/Cas9-mediated editing. The method may prove useful with other DNA viruses and in other biological systems.

**RESULTS**

**CRISPR/Cas9-mediated editing of the EBV genome in cultured human cells**

CRISPR/Cas9-mediated editing requires both gRNA and the protospacer adjacent motif (PAM) (Hsu et al., 2014). To construct a BART-deficient EBV using CRISPR/Cas9-mediated editing, we employed two gRNAs, pB1 and pB2, to target two sites flanking the entire pBART region that contains both P1 and P2 (Fig. 1a). PAMs required for target sequence identification and cleavage by the CRISPR/Cas9 system were found in the proximity of the target sites (Fig. 1b). HEK293-BX1 cells, which constitutively carry the GFP expressing BX1 strain of EBV and were established through co-culture with Akata-BX1 cells (Chen et al., 2005b; Tsang et al., 2010), were chosen as the target cells for CRISPR/Cas9 editing because HEK293 cells are highly transfectable, and have been extensively used for production and analysis of recombinant EBVs created on BACs (Seto et al., 2010; Geiser et al., 2011). The EBV genome copy number in HEK293-BX1 cells as analysed by fluorescence in situ hybridization (FISH) was 79 ± 52. To express Cas9 and the two gRNAs, we transiently transfected PX459-gRNA-pB1 and PX459-gRNA-pB2 plasmids into HEK293-BX1 cells. Total genomic DNA was analysed 2 days after transfection. A prominent DNA band consistent with targeted editing was observed when we PCR-amplified pBART region using two independent pairs of primers (Fig. 1c, lane 1 compared with lane 2). Judging from the relative band intensity, but taking transfection efficiency and the multicopy nature of the EBV genome into consideration, the Cas9-mediated cleavage was apparently efficient.

Inspired by these results, we expanded our analysis to other EBV-positive epithelial cells. In the study of EBV and its associated epithelial cancers, such as nasopharyngeal and gastric carcinoma, targeted disruption of specific viral genes in latently infected cells is highly desirable. Although a BAC clone of an epitheliotropic EBV strain was produced recently (Tsai et al., 2013), there is still a need to perform genetic analysis of additional strains, including C666-1 cells, which are highly representative of nasopharyngeal carcinoma (Cheung et al., 1999; Tso et al., 2013). It will thus be of great interest to see whether EBV genomes in these additional epithelial cells could be targeted by the CRISPR/Cas9 system. In light of this, we tested three EBV-positive epithelial cell lines, AGS-BX1, C666-1 and NP460-EBV, for susceptibility to CRISPR/Cas9 editing. AGS-BX1 and C666-1 are good models for EBV-associated gastric and nasopharyngeal carcinoma, respectively (Tso et al., 2013; Marquitz et al., 2014). NP460 is an immortalized nasopharyngeal epithelial cell line (Yip et al., 2013). Infection of NP460 with EBV through co-culture with infected B cells might shed light on early events of cancer
development (Tsang et al., 2010, 2012). It is noteworthy that C666-1 and NP460-EBV are difficult-to-transfect cells and that the copy numbers of the EBV genome (30–50 for C666-1 and 10–30 for NP460-EBV) in these cells are relatively high (Lun et al., 2012; Yip et al., 2013). The EBV genome copy number in AGS-BX1 cells as determined by FISH was 45 ± 33. When we expressed Cas9 and the two gRNAs in these cells, and harvested cells 2 days after transfection, targeted editing was seen in all three lines, albeit at relatively low levels (Fig. 1c, lanes 3–8). Our results indicated that CRISPR/Cas9-mediated editing was also feasible in these EBV-positive epithelial cells.

Fig. 1. CRISPR/Cas9-mediated editing of the EBV BART promoter in cultured human cells. (a) Schematic diagram of the 5′ part of the BART region in the EBV genome. The BART promoters (P1 and P2) are indicated and black arrows point to the target sites of gRNAs. Target sites of PCR primers p1F, p2F, p3F, p1R and p2R are also shown. (b) Sequence and binding sites of gRNAs in the EBV genome (GenBank accession number of EBV BX1: KC207813.1). The PAM motifs are highlighted in red. (c) CRISPR/Cas9-mediated editing. HEK293-BX1, AGS-BX1, C666-1 and NP460-EBV cells were transfected with PX459-gRNA-pB1 and PX459-gRNA-pB2 (gRNA1 +2). Cells were harvested 48 h after transfection, and total genomic DNA was extracted and analysed by PCR. Two pairs of BART promoter (pBART) primers p1 (p1F + p1R) and p2 (p2F + p2R) were used in the PCR that amplified both unedited (869 bp for p1 and 840 bp for p2) and edited (314 bp for p1 and 283 bp for p2) versions of the EBV genome. Genomic glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) was amplified as a control. (d) Clonal selection and expansion. After puromycin selection and recovery, clones were screened and a representative pBART-deleted clone (HEK293-BX1DpB) was verified by PCR. HEK293-BX1 cells mock transfected with empty PX459 vector and selected with puromycin (HEK293-BX1-mock) were analysed as a control. (e) Sequence of the edited EBV genome in one representative HEK293-BX1DpB clone.
Isolation of pBART-deleted EBV through puromycin selection

We sought to isolate the pBART-deleted EBV from the transfected HEK293-BX1 cells. As the Cas9/gRNA co-expression plasmid PX459 has a puromycin-selectable marker (Ran et al., 2013), we performed puromycin selection to enrich cells stably expressing Cas9 and gRNAs. Untransfected cells and cells that had lost the Cas9/gRNA co-expression plasmid were killed. Out of 50 clones of puromycin-resistant cells, three were found to be devoid of the unedited form of EBV (see Fig. 1d for one representative clone named HEK293-BX1ΔpB). DNA sequencing verified the deletion of the entire pBART region. In particular, a 558 bp deletion was found in the representative clone (Fig. 1e).

We went on to perform phenotypic verification of HEK293-BX1ΔpB cells. Quantitative real-time (qRT)-PCR was carried out to verify the expression of BART RNA and various miRNAs derived from BART RNA (miR-BARTs) as described previously (Choy et al., 2008a, b; Lei et al., 2013a, b). As one example, the expression of miR-BART3 was barely detectable in HEK293-BX1ΔpB cells (Fig. 2a). The trace amount of miR-BART3 in HEK293-BX1ΔpB may be due to detection error or leaky expression in the absence of promoter. Nevertheless, no miR-BART3 activity was detected with a reporter plasmid containing either perfect miR-BART3 targets (pmirGLO-miR-BART3) or the 3′ UTR of the miR-BART3-regulated gene DICE1 (pGL3-DICE1-3′UTR) (Fig. 2b). These constructs were fully characterized in our previous publications (Lei et al., 2013a, b). Similar results were also obtained for all other pBART-deleted clones and miR-BARTs analysed. Thus, we used a recombinant virus to provide the first genetic evidence that pBART containing both P1 and P2 is the main promoter for the expression of BART RNA, which gives rise to the miR-BARTs (Edwards et al., 2008).

We next induced lytic replication of pBART-deleted EBV and recovered virus from culture supernatant. The recovered virus was verified for the complete depletion of the pBART region. Genomic glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) was not detected, excluding the possibility of cell contamination (Fig. 3a). In addition, we PCR-amplified several regions of the viral genome, including pBART, EBER, LMP1, BZLF1 and the BamHI W region. All amplified fragments were of the expected size. In particular, the complete depletion of pBART was substantiated by the absence of amplification product when a primer targeting the deleted region (p3F) was used (Fig. 3a). Sequencing results confirmed the identity of all amplified fragments and no unwanted mutations were found. Moreover, no off-target mutations were detected by deep sequencing of EBV DNA. Except for the deletion of pBART and the presence of single nucleotide polymorphisms, the EBV sequence assembled was found to be identical to the recently reported genome sequence (GenBank accession number KC207813.1) of EBV derived from Akata cells (Lin et al., 2013). Although some ambiguous and unresolved stretches were left in the repeat regions, as in the reported sequence of BX1 and other EBV strains (Liu et al., 2011; Lin et al., 2013; Tso et al., 2013; Kwok et al., 2014), there was no evidence that the repeats were lost or significantly reduced in number. Furthermore, we verified the titre of the recovered virus on Raji and EBV-negative Akata cells (Fig. 3b, c). Notably, in both Raji and Akata cells, the viral yields of the pBART-deleted virus recovered from HEK293BX1ΔpB cells were lower than those of the WT virus recovered from HEK293BX1 cells (Fig. 3c). It is plausible that the deletion of pBART might affect virus production.
Isolation of pBART-deleted EBV through single-cell sorting

Amongst the 50 puromycin-resistant clones of HEK293-BX1 cells constitutively expressing Cas9 and gRNAs, 47 contained both edited and unedited EBV genomes. To determine the cleavage efficiency of Cas9 in these cells designated HEK293-BX1/BX1ΔpB, we compared the relative band intensities of edited and unedited EBV DNA. The DNA bands corresponding to the unedited form were faint in all 47 clones examined, suggesting that CRISPR/Cas9-mediated cleavage was fairly efficient. Data from two representative clones are presented in Fig. 4(a). To assess the amounts of unedited EBV DNA in HEK293-BX1 and HEK293-BX1/ΔpB cells so that the cleavage efficiency might be derived, we performed qRT-PCR with primers that specifically amplified only the unedited version of the EBV genome (Fig. 4b). The normalized amounts of unedited EBV DNA in these cells were then compared. In one representative clone (clone 1), the cleavage efficiency was 94.2%.

We next explored how the pBART-deleted recombinant EBV could be isolated from the puromycin-resistant HEK293-BX1/ΔpB cells. We recovered a mix of edited and unedited EBV viruses from clone 1 of HEK293-BX1/ΔpB cells induced to undergo lytic replication. EBV-negative Akata cells (Shimizu et al., 1996) were subsequently infected with the recovered viruses at a low m.o.i. The infected Akata cells were subjected to single-cell sorting. The single Akata cells recovered to 96-well plates were then analysed by nested PCR. Six out of eight cells contained pBART-deleted EBV only (Fig. 4c). Thus, recombinant EBV with the desired deletion could be isolated successfully through reinfection coupled with single-cell sorting.

Introduction of the DsRed marker during CRISPR/Cas9-mediated editing of EBV genome

To facilitate the isolation of mutant virus generated by CRISPR/Cas9-mediated editing, the insertion of a selectable marker is desirable. Double-strand breaks created by Cas9 can be repaired by both non-homologous end-joining and homologous recombination (Mali et al., 2013). Supplying a marker flanked by homology arms provokes homologous
recombination, resulting in marker insertion at the break (Hsu et al., 2014). To test the feasibility of marker insertion during the course of CRISPR/Cas9-mediated editing of the EBV genome, we PCR-amplified a DsRed marker flanked with 50 bp EBV homology arms, which were introduced by the PCR primers (Fig. 5a). We then co-transfected this DsRed DNA fragment together with the expression plasmids for Cas9 and the two gRNAs into HEK293-BX1 cells. In addition to the edited (314 bp) and unedited (869 bp) forms of the pBART amplification product, a weak band consistent with the insertion of DsRed (1850 bp) was also observed (Fig. 5b, upper panel). When we amplified with specific primers, a discrete DsRed band (1495 bp) was evident (Fig. 5b, lower panel). The mix of EBV viruses was then introduced into Akata cells as described above, and the GFP+ and DsRed+ recombinant virus was isolated by cell sorting. Sequencing results confirmed the replacement of pBART by the pCMV-DsRed cassette as desired (Fig. 5c). Hence, a selectable marker could be introduced successfully during CRISPR/Cas9-mediated editing of the EBV genome.

**DISCUSSION**

In this study, we demonstrated CRISPR/Cas9-mediated targeted editing of the EBV genome in cultured human cells. Under the direction of two gRNAs, the entire pBART region was deleted successfully, resulting in the loss of miR-BART expression and activity. This provides the first genetic evidence that pBART is the main promoter for BART expression. The editing was reasonably efficient and no off-target cleavage was noted. Recombinant viruses with the desired deletion were isolated through drug selection and single-cell sorting. Additionally, a selectable marker was introduced successfully during the course of CRISPR/Cas9-mediated targeting. Our work provides a new tool for the genetic study of EBV and other DNA viruses. The CRISPR/Cas9 technology may also prove useful in the development of vaccines as well as antiviral and anti-cancer agents.

The strategy to ablate miRNA expression and activity through the deletion of its promoter can be generalized and used in other biological systems. In this sense, our work provides an efficient and versatile method for miRNA ablation. Furthermore, our approach will find wide applications in many other contexts. Although our experiments were done in epithelial cells latently infected with EBV, the same method can, in principle, also be adopted with any other DNA viruses and any other cells. CRISPR/Cas9-mediated editing of adenoviral and HSV-1 genomes was recently reported (Bi et al., 2014). The present study and the one by Bi et al. (2014) corroborate with each other to indicate the susceptibility of episomal viral DNA to CRISPR/Cas9-mediated editing.

In contrast to the published work on adenovirus and HSV-1, which was done in acutely infected cells in which the virus undergoes lytic replication, our results were obtained...
from human cells persistently infected with EBV. Although the activation status of the viral genome in these two types of infected cells is very different, the viral DNA in both can be edited by CRISPR/Cas9. Whereas only one gRNA was used in the study by Bi et al. (2014), two gRNAs were used in our present work to direct the deletion of a sizeable DNA fragment. In this regard, our work expands the scope of application of the method in targeted editing of viral DNA. In addition, we described the isolation of pure recombinant virus by drug selection and by reinfection coupled with single-cell sorting. This isolation is required for phenotypic characterization of the edited virus. Finally, we reported the successful introduction of a selectable marker during CRISPR/Cas9-mediated editing of the EBV genome. This will significantly facilitate the isolation and subsequent characterization of the mutant virus.

Although our development of the method for RNA-guided editing of EBV genome was highly successful, our results also raised new questions and suggested several new directions for further investigators. First, the efficiency of CRISPR/Cas9-mediated editing in AGS1-BX1, C666-1 and NP460-EBV cells remains to be increased. The EBV genome copy numbers in these difficult-to-transfect cells are known to be high (Lun et al., 2012; Yip et al., 2013). One way to achieve high-efficiency gene transfer is to use retroviral and lentiviral vectors to deliver Cas9 and gRNAs. Second, although no off-target cleavage was detected by deep sequencing in our study, the nature and extent of off-target effects in CRISPR/Cas9-mediated editing of the EBV genome warrant further investigations. Off-target cleavage remains a major concern in the application of CRISPR/Cas9 technology (Cho et al., 2014). If necessary, Cas9 nickase might be used to minimize off-target effects (Ran et al., 2013; Shen et al., 2014). Extensive restriction mapping of the recombinant virus can help to assess the overall integrity of the EBV genome. However, a large amount of viral DNA is required for this analysis. Off-target effects on the cellular genome, particularly in cells constitutively expressing Cas9 and gRNAs, should also be taken into consideration. However, when that is a concern, the recombinant virus recovered could be used to infect other cells. Third, it will be of interest to clarify whether the trace amount of miR-BART3 that we detected in cells harbouring the pBART-deleted virus might be attributed to leaky expression or a detection error of the ultrasensitive qRT-PCR. As

Fig. 5. Introduction of DsRed during CRISPR/Cas9-mediated editing of the EBV BART promoter. (a) Schematic diagram of the 5’ part of the BART region in the EBV genome. The pCMV-DsRed fragment to be inserted is shown in red. Target sites of PCR primers p2F, DsR and p2R are also indicated. (b) DsRed insertion. The indicated combinations of DNA were transfected into HEK293-BX1 cells. Cells were harvested 72 h post-transfection, and the total genomic DNA was extracted and analysed by PCR. Primers p2F+DsR were used to amplify the pCMV-DsRed insert (1495 bp). (c) Sequence of the pBART-pCMV-DsRed junction in a mutant virus.
viruses (including EBV) are known to transcribe promiscuously (Liu & Speck, 2003), pBART-independent expression of miR-BART3 is not totally unexpected. We are currently in the process of deleting the complete BART region using CRISPR/Cas9 technology. Fourth, we found that the yield of pBART-deleted EBV is lower than that of the WT virus as measured in Raji and Akata cells. This might contribute partly to the low ratio of pure recombinants isolated. A dramatic difference was also seen when we infected nasopharyngeal epithelial cells with the same amount of the two viruses (data not shown). This pointed to the importance of miR-BARTs in viral infection of epithelial cells. Full documentation of this observation and biological characterization of pBART-deleted EBV in epithelial cells will be reported in our next paper. miR-BARTs are abundantly expressed in infected epithelial cells (Cai et al., 2006; Choy et al., 2008b). It will be of great interest to see whether they are particularly required for infection and transformation of epithelial cells. For comparison, miR-BHRF1s, which are expressed abundantly in infected B cells, have been shown to promote survival, proliferation and transformation of B cells (Seto et al., 2010; Feederle et al., 2011). The yield of miR-BART3-deleted EBV created by CRISPR/Cas9 technology was comparable with that of WT virus (data not shown). Thus, the low yield of pBART-deleted virus was unlikely due to CRISPR/Cas9-mediated editing per se. Finally, our work only provides the proof-of-principle and an example of RNA-guided editing of the EBV genome and the subsequent isolation of the EBV mutant of interest. Variations of the approach can be further developed to fit different purposes in the targeted engineering of EBV and other DNA viruses. For example, a combination of CRISPR/Cas9-mediated cleavage and PCR generation of homologous recombination donors, as demonstrated in our study for DsRed and elsewhere (Böttcher et al., 2014), might enable epitope tagging and insertion of a reporter gene, a drug selectable marker or a prokaryotic replication origin.

CRISPR/Cas9-mediated editing of the EBV genome in human cells provides a new technology platform for the genetic study of EBV. In particular, it will facilitate rapid analysis of the roles of individual EBV genes in viral replication, persistence and transformation. This method is still in its infancy and is evolving rapidly. Currently, the creation of point mutants and the isolation of pure mutants, particularly point mutants, are challenging. The off-target effects are another major concern. Compared with BAC clones, it will be more difficult to obtain large amounts of CRISPR/Cas9-edited viral DNA for the assessment of genome integrity by restriction mapping. Due to these and other deficiencies, CRISPR/Cas9-mediated editing cannot currently replace BAC recombining in the genetic study of EBV. However, the new method also has several advantages and is highly complementary to the existing BAC technology. First, CRISPR/Cas9 technology is applicable to any EBV strain, whereas EBV BACs are currently available for only three strains (Westphal et al., 1998; Kanda et al., 2004; Chen et al., 2005a; Sato et al., 2010; Tsai et al., 2013). Second, CRISPR/Cas9-mediated editing is performed completely in human cells, whereas EBV BACs are constructed and produced in Escherichia coli. The influence of the bacterial type of methylation pattern on EBV gene expression upon subsequent introduction of BAC into human cells is therefore prevented. Third, operation of the CRISPR/Cas9 system does not require the insertion of an additional sequence into the EBV genome. In contrast, prokaryotic F factor has to be inserted into the EBV genome in EBV BAC. This insertion might cause unexpected deleterious effects. For example, an F factor was inserted into the BART region in one version of EBV BAC (Sato et al., 2010), preventing its use in the analysis of BART function.

Finally, as shown in our study, virus-producing cell lines for CRISPR/Cas9-edited EBV can be readily made with EBV-negative Akata cells, which enable high-yield production of EBV (Shimizu et al., 1996). In the case of EBV BAC, the creation of high-quality virus-producing cells is considered a bottleneck (Feederle et al., 2010). For all these reasons, CRISPR/Cas9 technology is a powerful and versatile tool in targeted engineering of EBV and other DNA viruses with a large genome.

METHODS

Plasmids. pmiRGLO-BART3 and pGL3-DICE1-3'UTR reporter plasmids have been described previously (Lei et al., 2013a, b). Whereas pmiRGLO-BART3 contains four copies of sequence perfectly complementary to miR-BART3 in the 3' UTR of the firefly luciferase gene, pGL3-DICE1-3'UTR carries the 3' UTR of the DICE1 gene.

gRNA-Cas9 co-expression plasmid PX459 (Ran et al., 2013) was a gift from Dr Feng Zhang (Massachusetts Institute of Technology, Cambridge, MA, USA). PX459-gRNA-pB1 and PX459-gRNA-pB2 were made by inserting the respective gRNA sequences. Target sites and sequences of gRNA-pB1 and gRNA-pB2 in the EBV genome are shown in Fig. 1(a, b).

The expression plasmid for Zta has been described previously (Sarisky & Hayward, 1996). The expression plasmid for gp110 was based on pHingen, and gp110 was PCR-cloned from genomic DNA of C666-1 cells using primers 5'-GAATTCATGACTCGGCGTAGGCGTGC-3' and 5'-GGGCGCGCCTTTAAACATCAGTCCTCCTCGCTCC-3'.

Cell culture and transfection. HEK293-BX1 cells were established by co-cultivation of EBV-infected Akata-BX1 with HEK293 cells as described previously (Chen et al., 2005b; Tsang et al., 2010). EBV-infected HEK293-BX1, gastric adenocarcinoma cell line AGS-BX1 (Marquitz et al., 2014), nasopharyngeal carcinoma cell line C666-1 (Cheung et al., 1999) and nasopharyngeal epithelial cell line NP600-EBV (Yip et al., 2013) were cultured and transfected as described previously (Choy et al., 2008b). C666-1 and NP600-EBV cells were transfected with TransIT-Keratinocyte transfection reagents (Mirus). Raji and EBV-negative Akata cells were cultured as described previously (Shimizu et al., 1996; Choy et al., 2008b). The EBV genome in infected cells was analysed by FISH, as described previously (Tsang et al., 2012). The mean ± SD copy number was obtained from 50 cells at metaphase.

CRISPR/Cas9-mediated genome editing. HEK293-BX1, AGS-BX1, C666-1 and NP600-EBV cells were transiently transfected with PX459-gRNA-pB1 and PX459-gRNA-pB2. For clonal selection and expansion, cells were trypsinized and transferred onto 10 cm dishes 72 h after transfection. As PX459 plasmid has a puromycin marker,
cells transfected with PX459-gRNA-pB1 and PX459-gRNA-pB2 were selected by 3 µg puromycin ml⁻¹ (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) for 3 days. Puromycin was then removed and cells were allowed to recover for a further 7 days. Clones plausibly derived from stably transfected cells appeared and were picked by a filter paper pre-soaked with trypsin/EDTA. The paper with cells attached was then transferred to a six-well plate filled with DMEM. Cells were allowed to recover and grow for a further 4 days. For introduction of DsRed, the pCMV-DsRed fragment containing the cytomegalovirus promoter (pCMV) was PCR-amplified from plasmid pDsRed2-C1 (Clontech). Primers with an EBV homology arm of 50 bp (5'-ATGGATTTGCTCGTCTTCCCTGGAGATAAATGGAGGACCCCGCTTTCCTGGATATCC-3' and 5'-TCTGCTGATGGACCCGTTCCTTCTCCGCTGAGTCATCCTAAGAGGGTTGCGAATTC-3'), the EBV homology arm is underlined) were used for the amplification. gRNA-Cas9 co-expression plasmids, PX459-gRNA-pB1 and PX459-gRNA-pB2, as well as the pCMV-DsRed fragment were co-transfected into HEK293-BX1 cells.

**PCR analysis.** Total genomic DNA was purified using a Wizard Genomic DNA extraction kit (Promega). EBV genomic DNA was purified using a MagMax nuclear acid isolation kit (Life Technology). Six primers (p1F: 5'-TCTCTCTACCTAATGGTCGAC-3'; p2F: 5'-GCCCTCTTACTCACAT-3'; p3F: 5'-GACGAGGTTGGATGATC-3'; p1R: 5'-GGATACGTGCAGTCGTAATG-3') were used in different combinations in PCR amplification of pBART for 32 cycles. Primers for amplification of the gGAPDH gene were as described previously (Lin et al., 2013). Primers for LMP1 were 5'-GGGCGCCATAGGTT-3' (forward) and 5'-GACATCTCTTGCTGAGTCCTCT-3' (reverse). Primers for BZLF1 were 5'-GCCACCTTTGCTTTGCTGCT-3' (forward) and 5'-TCTTCTCTATGACAGCACC-3' (reverse). Primers for EBER and the BamH I W region were as described previously (Chen et al., 2005b). qRT-PCR was performed using a SYBR Premix Ex Taq system (TaKaRa) as described previously (Kew et al., 2013; Siu et al., 2014).

For single-cell PCR, single GFP⁺ Akata cells were sorted by a FACSAria SORP cell sorter (BD Bioscience) into 96-well plates filled with 5 µl lysis buffer (0.1 % NP-40, 0.1 mM dNTP and 10 mM DTT). PCR mix (45 µl) was added to each well for the first round of PCR with primers p1F and p2R for 40 cycles. An aliquot of 5 µl of the product was subjected to a second round of PCR with primers p2F and p1R for another 40 cycles. The identity of PCR products was confirmed by direct DNA sequencing. Deep sequencing of the complete EBV genome was performed on an Illumina HiSeq 1500 sequencer as described previously (Tso et al., 2013; Kwok et al., 2014). Briefly, EBV genomic DNA was extracted and DNA libraries were prepared using protocols suggested by Illumina. The mean fragment size was ~250 bp. Paired-end sequencing with read lengths of 150 bp was performed. The raw data were filtered using Illumina quality control parameters. The assembled EBV sequence was compared with the reference genome sequence (GenBank accession number KC207813.1) of EBV derived from Akata cells (Lin et al., 2013). The aligned sequence covered 99.9 % of the EBV genome.

**qRT-PCR analysis of miRNA.** Total RNA was treated with DNase I. cDNA was synthesized by using a miRCURY LNA Universal RT microRNA PCR kit (Exiqon). cDNA diluted in nuclease-free water was added to SYBR Green Master Mix. For qRT-PCR of miR-BART3-3p (5'-CCGAGGCAGGUGU-3'), specific LNA (locked nucleic acid) primers were ordered from Exiqon. qRT-PCR was carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems). Normalization was made to housekeeping RNA SNORD44.

**EBV infection of Raji and Akata cells.** EBV-infected HEK293 cells (1 × 10⁶) were seeded into 10 cm dishes and transfected with 4 µg each of pgp110 and Zta expression plasmids to induce lytic replication. Culture supernatant was collected 72 h post-transfection. Raji or EBV-negative Akata cells (1 × 10⁵) were infected with 500 µl EBV-containing culture supernatant. Cells were placed in 24-well plates with 1 ml RPMI 1640 medium in each well. After 1 day of infection, culture medium was replaced with 1 ml fresh RPMI 1640 medium. Cells were incubated for an additional 2 days. Three days after infection, Raji cells were examined by fluorescence microscopy and single Akata cells were sorted by using a FACSAria SORP cell sorter (BD Bioscience). Viral titres were measured by green Raji or Akata units calculated from the percentages of green cells × 10⁻².

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**REFERENCES**


