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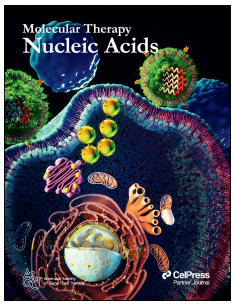

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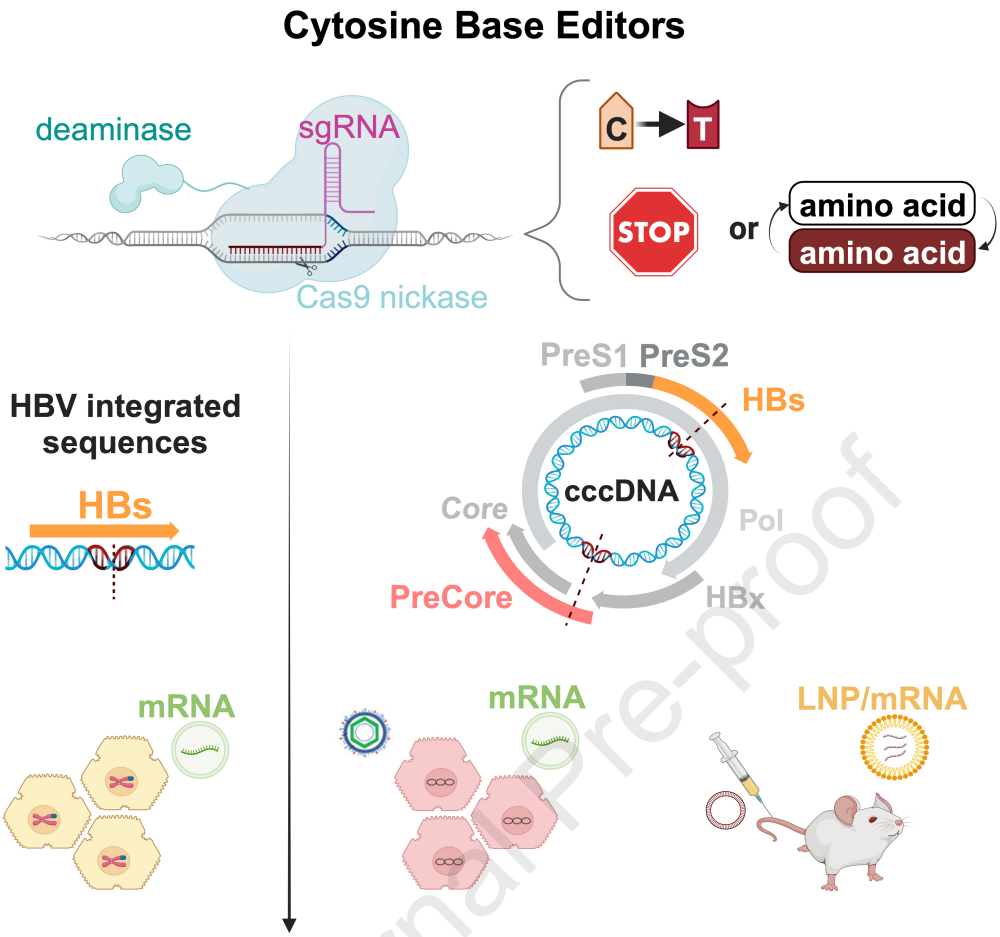

Cytosine base editing inhibits Hepatitis B Virus replication and reduces HBsAg expression *in vitro* and *in vivo*

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Short title

Cytosine base editing to inactivate HBV genome

Abstract

Chronic Hepatitis B virus (HBV) infection remains a global health problem due to the lack of treatments that prevent viral rebound from HBV covalently closed circular (ccc)DNA. Additionally, HBV DNA integrates in the human genome serving as a source of hepatitis B surface antigen (HBsAg) expression, which impairs anti-HBV immune responses. Cytosine Base Editors (CBEs) enable precise conversion of a cytosine into a thymine within DNA. In this study, CBEs were utilized to introduce stop codons in HBV genes, *HBs* and *Precore*. Transfection with mRNA encoding a CBE and a combination of two guide RNAs led to robust cccDNA editing and sustained reduction of the viral markers in HBV infected HepG2-NTCP cells and primary human hepatocytes. Furthermore, base editing efficiently reduced HBsAg expression from HBV sequences integrated within the genome of PLC/PRF/5 and HepG2.2.15 cell lines. Finally, in the HBV minicircle mouse model, using lipid nanoparticulate delivery, we demonstrated antiviral efficacy of the base editing approach with a >3log10 reduction in serum HBV DNA and >2log10 reduction in HBsAg, and 4/5 mice showing HBsAg loss. Combined, these data indicate that base editing can introduce mutations in both cccDNA and integrated HBV DNA, abrogating HBV replication and silencing viral protein expression.

Introduction

Chronic Hepatitis B (CHB) remains a global health problem with more than 250 million people infected with hepatitis B virus (HBV) worldwide.[1–3] It is estimated that 30% of patients develop hepatocellular carcinoma and cirrhosis, which leads to 800,000 deaths per year.[4,5] The HBV genome is maintained in the hepatocyte nucleus as a 3.2kb episomal covalently closed circular DNA (cccDNA), which is the source of continuous viral replication.[6,7] While standard-of-care antivirals (nucleos(t)ide analogs, NAs) efficiently inhibit viral replication, they do not affect cccDNA, which persists in the liver during lifetime of a patient, preventing cure of CHB.[5] In addition to being a persistent reservoir of cccDNA, HBV DNA sequences are known to integrate into the human genome. These copies of integrated HBV DNA can serve as a template for up to 80% of *HBs* gene transcripts in the late phase of infection.[8] The resulting expression of Hepatitis B surface antigen (HBsAg) impairs host immune responses against the virus and contributes to the persistence of HBV.[8–10] Therefore, novel classes of drugs that could inactivate both cccDNA and integrated HBV DNA are needed to enable a cure for CHB.[7]

Gene editing technologies have the potential to directly target and inactivate both aforementioned viral DNA species. Although nuclease gene editing strategies have been shown to reduce cccDNA levels within *in vitro* and *in vivo* models[11,12] , one concern with this approach is the generation of double-strand break (DSB) intermediates [13] Given that HBV DNA can integrate at multiple locations in the human genome within a single cell, gene editing with a nuclease targeting an HBV DNA sequence could simultaneously generate DSBs at multiple genomic loci leading to undesirable genomic rearrangements.[14,15] Unlike nucleases, base editors perform a chemical reaction, deamination, thus converting one nucleotide into another without the need for a DSB intermediate.[16,17] Base editing enables multiple edits in a single cell with high efficacy and minimal

genomic rearrangements, compared to CRISPR-Cas9.[18] Cytosine base editors (CBEs) convert cytosine into thymine, enabling gene silencing through introduction of the stop codons, an approach that previously showed promise for targeting HBV DNA in cell models.[16,19,20] While previous studies[19,20] demonstrated base editing of HBV sequences and silencing of viral gene expression, the utilized models were exclusively in vitro systems not suitable to assess base editing on an established cccDNA pools characteristic of CHB.

In this study, we have identified a combination of two guide RNAs (gRNAs), that, when paired with a CBE, inactivated both cccDNA and integrated HBV DNA in relevant HBV cell models, including HBV-infected HepG2-NTCP, HBV-infected primary hepatocytes (PHHs), as well as HepG2.2.15 and PLC/PRF/5 cell lines with artificially and naturally integrated HBV DNA, respectively. Furthermore, for the first time, we have shown durable antiviral efficacy, including HBsAg loss, *in vivo* in the HBVcircle mouse model using lipid nanoparticulate (LNP) delivery of base editing reagents (mRNA/gRNA). Combined with a thorough evaluation of gRNA-dependent off-target effects, this data advances our understanding of the potential of base editing to enable a functional cure for chronic HBV infection.

Results

HBV gRNA design and screen

HBV genotype D (subgenotype ayw) was used for gRNA design, as this is a common genotype used in many cellular and animal models of HBV infection, therefore extensive sequence data are available.[21] Two different strategies were used to design gRNAs. In the first strategy, we used the Benchling CRISPR tool[22] to identify 33 gRNAs that when paired with a prototypical CBE, BE4,

would be expected to introduce stop codons in the four main HBV genes: *Polymerase*, *HBs*, *(Pre)Core*, and *X*. In the second strategy, we identified the gRNAs exhibiting high target site conservation across HBV isolate sequences in the HBVdb database[23] that, in combination with the cytosine base editor, were predicted to introduce missense mutations in HBV genes **(Tables S1-2)** if C:G to T:A substitutions were made within the editing window of BE4**.** To test the editing efficacy of the gRNAs, we generated HEK293T cell lines with integrated HBV DNA sequences. Briefly, HEK293T cells were transduced with a lentivirus carrying HBV DNA sequences 2309-1622 (*HBs*, *Pol*) or 1176-2451 (*X*, *Core*). The resulting cell lines were transfected with the two plasmids encoding base editing reagents: BE4 and a gRNA. The gRNAs were then ranked based on two factors: (1) the rate of editing outcomes encoding stop codons or missense mutations as assessed by next-generation sequencing (NGS) and (2) the conservation across HBV genotypes **(Tables S1-2)**.

Base editing with selected gRNAs suppresses HBV viral parameters in *de novo* infected HepG2-NTCP cells

We examined the effect of BE4 with the six selected gRNAs (**Figure S1A**) on HBV parameters in HBV infected HepG2 cells stably expressing the hNTCP receptor, i.e. HepG2-NTCP cells.[24] RNA transfection methods have proven to be more efficient than DNA transfection, and less toxic for cells.[25] Therefore, RNA transfection was chosen to test selected gRNAs in HBV infected HepG2-NTCP cells. Protocols were optimized to efficiently deliver BE4 mRNA and gRNAs to study the effect of editing on the replicative ability of *de novo* established cccDNA (**Figure S1B, 2A)**.[26] Levels of extracellular HBs antigen (HBsAg) and HBe antigen (HBeAg) were measured in supernatants by ELISA, while total HBV DNA and 3.5kb RNA were quantified intracellularly

using qPCR and RT-qPCR, respectively. Several of these treatment groups exhibited reduction of selected HBV viral markers (**Figure S1C-F**). In particular, gRNAs g37 (designed to introduce a stop codon in *HBs* gene) and g40 (designed to introduce a stop codon in *Precore* gene) (**Figure 1A**) drastically reduced HBsAg and HBeAg, respectively **(Figure S1C,D and 2B,C)**. Interestingly, inhibition of total HBV DNA and 3.5kb RNA upon treatment with g37 and g40 was also observed **(Figure S1E,F and 2D,E)**. Further, to increase the suppression of all four viral parameters, we combined the two gRNAs (g37+g40) and successfully achieved reduction of HBsAg, HBeAg, total HBV and 3.5kb RNA **(Figure 2B-E).** The reduction in 3.5kb RNA was confirmed by Northern Blot, along with a reduction in 2.4kb and 2.1 kb HBs mRNAs, though the effect was less pronounced for these shorter HBV mRNA species **(Figure S2A).** At the intracellular protein level, a decrease in all three HBs isoforms (L, M, and S) upon g37+g40 treatment was demonstrated by Western blot **(Figure S2B).**

HBV DNA intermediates (e.g., cccDNA, protein free (PF)-rcDNA, rcDNA) share high sequence similarity and gRNA/BE4 complexes could possibly target any of these DNA species, if the recognition sequence is present as dsDNA. We therefore investigated the direct impact of gRNA/BE4 editing on cccDNA by pretreating infected cells with 2′,3′-didéoxy-3′-thiacytidine (3TC or lamivudine), which reduced the amount of viral DNA replicative intermediates with respect to cccDNA at the time of base editing **(Figure S3A).** Similar to 3TC untreated condition (as shown in **Figure 2**), reduction in viral parameters was observed after the transfection with BE4 and g37, g40, or (g37+g40) **(Figures S3B-S3F)** and no difference in BE4 expression was observed in 3TC untreated or treated conditions **(Figure S3G).** These results strongly suggest that the reduction in viral replicative parameters is a consequence of base editing direct effect on cccDNA.

We next assessed cccDNA levels by two methods: cccDNA-specific qPCR on the total DNA samples treated with ExoI/III nucleases (**Figure 2F and S3H**) and Southern blot analysis on Hirt extracted samples followed by ExoI/III digestion (**Figure S4A**) to decrease the levels of HBV replicative intermediates other than cccDNA.[27] Densitometry analysis of Southern Blot bands and qPCR consistently showed no difference in cccDNA levels in edited samples.

We further performed NGS on total DNA samples treated with ExoI/III nucleases to assess cccDNA editing rates that led to the introduction of the Stop codons in *HBs* and *Precore* genes in the presence or absence of 3TC. Treatment of HBV-infected HepG2-NTCP cells with BE4/g37 led to C7T edit, successfully introducing W156Stop amino acid change in *HBs* gene. Similarly, treatment with BE4/g40 led to C8T edit, resulting in W28Stop change in *Precore* gene. Multiplexing BE4 and gRNAs g37+g40 led to editing of both C7T (*HBs*) and C8T *(Precore*) sites (**Figure 2G**). High levels of editing at all positions were maintained in the case of the combinatorial treatment with 3TC (**Figure S3I**). NGS on the Hirt extracted samples followed by ExoI/III digestion that were analyzed by Southern Blotting (**Figure S4A**) also confirmed a very high editing efficiency in the presence or absence of 3TC (48-70% C7T for *HBs* gene and 60-70% for *Precore* gene) (**Figure S4B**).

Taken together, these data demonstrate that BE4 mediates inhibition of HBV replication and viral antigens production in infected HepG2-NTCP cells. Combination with 3TC showed that this inhibition is maintained in the context of reduced replicative intermediates, suggesting direct base editing and introduction of stop codons in cccDNA.

Antiviral efficacy of base editing in HBV-infected primary hepatocytes (PHH)

Primary human hepatocytes (PHHs) isolated from chimeric mouse liver allow to maintain hepatocyte differentiation and HBV replication for up to 30 days.[28,29] These PHHs were infected with HBV for at least 4 days to allow generation of a stable pool of cccDNA before transfection. Two consecutive transfections with mRNA and gRNA (day 5 and day 12 after HBV infection) were performed to increase efficacy. After the last transfection, PHHs were kept in culture for two weeks, and the experiment terminated 25 days post infection (**Figure 3A**). Consistent with the data in HepG2-NTCP cells, transfection with BE4 and individual gRNAs led to reduction in the respective viral markers, while the combination of BE4 with both g37 and g40 resulted in simultaneous reduction in HBsAg, HBeAg, intracellular HBV total DNA and 3.5kb RNA (**Figure 3B and S5A**). Albumin expression level was similar in the control and treatment conditions confirming that PHH functionality was not compromised by HBV targeting gRNAs (**Figure S5B**). We have further assessed extracellular HBV DNA load in the supernatant over the course of the experiment. In this case, the treatment with BE4/(g37+g40) was compared to the temporary 3TC treatment. As expected, 3TC efficiently reduced HBV replication, however after treatment discontinuation, HBV DNA levels increased indicating viral rebound. Contrary to 3TC, there was no rebound in the case of treatment with BE4/(g37+g40) (**Figure 3C**) suggesting that base editing efficiently reduces HBV replication and prevents viral rebound.

BE4 protein expression from the transfected mRNA was transient and could not be detected by 24 hours post transfection (**Figure S5C**), suggesting that the observed antiviral efficacy resulted from permanent changes in HBV cccDNA rather than transcriptional interference of the BE4 protein with the viral genome.

Similar to HepG2-NTCP, cccDNA level did not change in HBV-infected PHH after the treatment with the base editing reagents (**Figure 3D**). In PHHs, two transfections with BE4/(g37+g40)

resulted in 59% cccDNA editing at g37 site (*HBs*) and 81% cccDNA editing at g40 site (*Precore*) (**Figure 3E**).

Expression of cellular deaminases is known to generate uracils within cccDNA that are processed by uracil glycosylase into abasic sites, ultimately leading to cccDNA degradation.[30] Although CBEs might also generate uracil intermediates within cccDNA, BE4 contains a uracil glycosylase inhibitor (UGI) domain, which inactivates base excision repair and thus increases the efficiency of the base editing.[16] To assess whether base editing could promote uracil glycosylase-induced cccDNA degradation, we transfected BE4 base editor without UGI (BE4_noUGI) with gRNAs 37 and 40 in HBV infected PHH. Similar to BE4, BE4_noUGI with g37 or g40 reduced HBV viral parameters and enabled robust cccDNA editing but did not affect cccDNA levels (**Figure S5D-S5E**). This result supports the concept that antiviral efficacy of base editing primarily functions through the introduction of nucleotide changes within cccDNA and not through any changes in cccDNA stability, at least under the tested PHH *in vitro* experimental conditions.

We have further assessed editing and antiviral efficacy of the next generation cytosine base editors BE4-PpAPOBEC1 and CBE-T. While displaying high on-target activity, they both exhibit minimal guide-independent off-target effects associated with cytosine deamination on cellular RNA and genomic DNA.[31,32] Two transfections with the combination of gRNAs (g37+g40) and one of the editors (BE4, BE4-PpAPOBEC1, or CBE-T) were performed in HBV-infected PHH. Both BE4-PpAPOBEC1 and CBE-T were efficient in reducing the four assessed viral markers: HBsAg, HBeAg, 3.5kb RNA, and HBV DNA (**Figure S6A**). BE4-PpAPOBEC1 and CBE-T were less efficient than BE4 in editing both HBs (46% and 36%, respectively) and Precore (62% and 28%). The level of editing correlated with the level of HBsAg and HBeAg reduction (**Figure S6A-**

B). These results show the broad applicability of different CBEs to directly edit the HBV genome and inhibit viral replication and antigen production.

CBE inhibits HBsAg expression from integrated HBV *in vitro*

Integrated HBV DNA is a source of HBsAg, which could represent most of the antigen production in late stages of CHB.[8,33,34] Therefore, we next examined if HBs targeting gRNA, g37 and the combination (g37+g40) were able to suppress the expression of HBsAg from integrated HBV genomes. For this, we used HepG2.2.15 cells harboring artificially integrated replication competent dimeric HBV genomes[35] , which were transfected with BE4 mRNA and g37 **(Figure 4A).** These cells were treated with 3TC to reduce the abundance of HBV DNA replicative intermediates that could compete as base editor substrates. On the 6th day after transfection, a strong decline in extracellular and intracellular HBsAg protein levels was observed in HepG2.2.15 cells treated with BE4 and g37 or (g37+g40) **(Figures 4B and S7).** Base editing efficiency was assessed by next generation sequencing of extracted genomic DNA, which revealed 60% C-to-T editing at the *HBs* target site in the samples treated with either g37 or (g37+g40) **(Figure 4D)**. Consistent with the results in HBV-infected HepG2-NTCP, targeting integrated *Precore* gene with g40 resulted in the reduction in extracellular HBeAg level in HepG2.2.15 cells, along with ~70% C8T edit introducing premature stop codon in *Precore* **(Figures 4C-4D).**

In addition, PLC/PRF/5 cells[36,37] were also used to test the efficiency of CBE to inactivate HBsAg from replication-incompetent naturally integrated HBV genotype A DNA sequences **(Figure 4E).** Upon Sanger sequencing, we observed a mismatch in g37 binding site in genotype A *HBs* gene compared to ayw genotype D of HepG2.2.15. Therefore, a gRNA compatible with the HBV sequence in PLC/PRF/5, represented as g37-PLC, was designed. Similar to the effect of g37 in HepG2.2.15, g37-PLC reduced the amount of secreted HBsAg **(Figure 4F).** A rate of 45% editing

was sufficient to observe a robust anti-HBs effect in this cellular model **(Figure 4G).** Taken together, we demonstrated that introduction of stop codons by cytosine base editing inhibits HBsAg expression produced from the integrated HBV sequences in cell lines with either artificially or naturally integrated HBV DNA.

Base editing leads to sustained reduction of HBV viral markers *in vivo*

To test antiviral efficacy of base editing *in vivo*, we used the HBV minicircle mouse model. This *in vivo* model supports persistent HBV replication and expression of viral antigens resulting from hydrodynamic injection (HDI) with a cccDNA-like plasmid.[38] Four weeks after HDI, the mice secreting HBsAg were organized into four groups. Hepatic delivery of base editing reagents was achieved via systemic administration of the lipid nanoparticles (LNP) formulated with mRNA encoding BE4 and control PCSK9 gRNA[39] or HBV-targeting gRNAs (g37+g40). Mice received one intravenous injection with LNP; after the injection, serum HBsAg, HBV DNA, and HBeAg levels were assessed weekly. Entecavir (ETV) treated mice received antiviral alone orally for two weeks, then the treatment was discontinued (**Figure 5A**). Six weeks after beginning of the treatment, we detected more than 2Log10 mean serum HBsAg reduction in the mice treated with BE4/(g37+g40); four out of five mice showed HBsAg reduction below the limit of detection (**Figure 5B and S8A-B**). Treatment with HBV-targeting base editing reagents further led to a sustained reduction in serum HBV DNA with no HBV viral rebound observed, compared to the entecavir group, in which serum HBV DNA was reduced following administration, but rebounded after entecavir treatment was discontinued (**Figure 5C and S8C-D**). Two weeks after treatment, loss of expression of the viral marker HBeAg was observed in all mice that received HBV-targeting base editing reagents (**Figure 5D and S8E-F**). Six weeks after the beginning of treatment, the study was terminated, and total HBV DNA level was assessed in mice livers.

Compared to controls, there was a decrease in HBV DNA amount in BE4/gRNAs(37+40) treated mice (**Figure 5E**). NGS showed that *in vivo* BE4/g37 introduced stop codon W156* in *HBs* gene with approximately 30% efficacy; BE4/g40 introduced stop codon W28* in *Precore* gene with 42% efficacy (**Figure 5F**). Taken together, this is the first demonstration of LNP-mediated delivery of base editing reagents targeting HBV sequences, showing sustained reduction of HBV parameters *in vivo*.

Off-target editing assessment

To evaluate gRNA-dependent off-target effects we performed RNase H-dependent amplification and sequencing (rhAmpSeq) analysis[40,41] of the *in silico* predicted off-target sites on the DNA samples derived from HBV-infected PHH transfected with the gRNAs (g37+g40) and cytosine base editors BE4, BE4-PpAPOBEC1, and CBE-T. Briefly, guide-dependent off-target candidates are identified *in silico* by running Cas-OFFinder[42] on the GRCh38/hg38 reference genome using the protospacer and NRR PAM specificity as input. Candidate off-target sites were stratified by their genomic location and PAM sequence. All sites with ≤3 mismatches to the on-target locus were included in the rhAmpSeq panel for evaluation in edited cells. Candidate off-target sites with 4 to 7 mismatches from the on-target that overlapped annotated exons or were within 100bp of an exon were also included. In this study, we have screened 499 and 685 potential off-targets in rhAmp-seq panel of g40 and g37, respectively. Each base in a candidate off-target site was compared between treated and untreated samples. A Fisher's Exact test was used to generate a significance score for enrichment of off-target edits in treated cells when compared to untreated cells.

The number of the identified off-target sites correlated with the level of on-target editing (BE4 > BE4-PpAPOBEC1 > CBE-T) (**Table S3**). Each off-target site was further characterized by its

genomic location (**Table S4**). Standard BE4 base editor with g37 and g40 resulted in 19 and 7 off-target sites, respectively. Majority of the off-target sites were located in the noncoding regions of the genome, namely intronic and intergenic regions, except for the two sites: [g37] in the BCL7A gene (non-synonymous mutation with 0.45% off-target editing) and [g40] in lncRNA gene AC131254.1. Next-generation CBE BE4-PpAPOBEC1 exhibited a more advantageous off-target profile (8 sites with g37, and one site with g40). CBE-T treatment was associated with the lowest off-target editing: HBs/Pol-targeting g37 yielded two off-target sites in the non-coding regions of the genome, and none were identified for the Precore-targeting g40.

Discussion

Currently available NA therapies, approved for CHB patients, suppress HBV replication but do not target HBV cccDNA or HBs expression from the integrated DNA sequences and hence do not cure CHB. The persistence of highly stable HBV cccDNA in infected hepatocytes leads to viral relapse upon discontinuation of therapy, and therefore life-long NA treatment is necessary. Undoubtedly, there is an urgent need for HBV therapeutics that target the HBV genomic reservoir (cccDNA) and integrated HBV DNA to avoid the need for long-term treatment.[1,5,7]

In recent years, several research groups applied CRISPR/Cas9 nuclease technology to eliminate intrahepatic HBV genomes.[11,13,26] However, this approach suffers from several limitations. For example, DSBs generated upon dsDNA cleavage by wild-type Cas9 endonuclease can lead to host genomic instability. Moreover, upon multiplexing, shorter episomal cccDNA variants could be generated that remain transcriptionally active.[26] In this study, we demonstrate the potential of base editing technology to irreversibly silence cccDNA and integrated HBV DNA, which was associated with the reduction/loss of the viral markers.

We have examined the utility of cytosine base editors (BE4) for inactivation of HBV cccDNA and integrated DNA templates. To do this, we screened forty different gRNAs and identified a combination of two that enabled simultaneous reduction of all four tested HBV markers (HBsAg, HBeAg, HBV DNA, and 3.5kb RNA) in HepG2-NTCP, as well as in PHH. We further showed that BE4/(g37+g40) mediated editing of HBV genomes not only reduced HBV replication in HepG2-NTCP and PHHs but also reduced HBsAg expression in HepG2.2.15 and PLC/PRF/5 cells, which harbor artificially and naturally integrated HBV DNA, respectively.

The high rates of base editing observed in samples from which HBV DNA replicative intermediates were removed using nucleases digestion[27] suggests that base editing antiviral efficacy is mediated by editing of cccDNA. To further test this hypothesis, infected HepG2-NTCP cells were treated with 3TC, which decreases the abundance of replicative intermediates, leaving predominantly cccDNA as a substrate for base editing. The reduction of viral parameters and even higher editing rates under this experimental condition demonstrates that cccDNA can be directly targeted by BE4. Furthermore, in clinical trials novel anti-HBV therapeutics are often added to the standard regimen of approved NA antivirals, such as 3TC.[1] Therefore, it is encouraging that base editing efficacy in a relevant HBV cell model was not compromised when combined with 3TC treatment.

Two previous studies have shown the potential of cytosine base editing to target HBV genomes, however, these studies utilized lentiviral-based delivery of base editors.[19,20] Lentiviral transduction with the base editing reagents performed prior to the infection does not allow establishment of cccDNA, and therefore has limited relevance to CHB. Here, we demonstrated delivery of gRNAs and an mRNA encoding a cytosine base editor in infection models with established cccDNA pool, including *in vivo* in an HBV mouse model.

As a delivery method we have utilized LNPs. This delivery strategy enables transient protein expression from short-lived mRNA[43], limiting exposure of the genome to the gene/base editor, which is associated with a more favorable off-target profile[44–46]. In this study, we examined for the first time *in vivo* efficacy of the base editing approach for inhibiting viral replication in HBV minicircle mouse model. This immunocompetent mouse model supports high levels of HBV replication and persistence. Contrary to HBV AAV mice, HBV replication in this model is driven directly from the cccDNA-like viral genomes. Hence, it is more physiologically relevant to test the efficacy of cccDNA targeting drugs or cccDNA-related processes.[38] Further, HBV replication recovery after discontinuation of entecavir showed that HBVcircle mice enabled rebound of the virus under the analyzed experimental conditions, indicating relevance of the model. Contrary to entecavir, the LNP-mediated hepatocellular delivery of BE4 mRNA and (g37+g40) led to a sustained reduction of not only serum HBV DNA, but also of secreted HBsAg in mouse serum for six weeks after LNP injection. For secreted HBeAg, we observed a drastic reduction below the limit of detection at an earlier time point (2 weeks post-injection). This dramatic reduction in viral antigens was accompanied by a partial editing of the cccDNA pool (30% and 40% for g37 and g40 target regions, respectively), which could be explained by the fact that editing would be targeting preferentially the transcriptionally active cccDNA molecules, which are more accessible to the editor.[47] As HBsAg and HBeAg play important roles in establishing and maintaining chronic infection through immunomodulatory effects, this approach could have additional benefit by restoring a more active immune environment in patients.[48] The sustained reductions in viral markers after a single administration of base editing reagents in this model reinforces the unique and potentially advantageous mechanism of gene editing relative to other modalities like siRNA or ASOs which require repeated administrations.[49,50]

Due to the overlapping nature of HBV genome, nonsense mutation in an ORF can introduce missense mutations in another ORF and/or affect a regulatory region important for viral gene expression/ replication. For example, base editing with g40 not only results in W28* in precore but also introduces mutations into the epsilon encoding region. As epsilon plays an important role in pgRNA encapsidation[51] , it is possible that mutating this site impacts pgRNA packaging and reduces HBV replication, as observed in this study. Similarly for g37, the W156* mutation in HBs also corresponds to a G500N amino acid substitution in the polymerase. Though the effect of this mutation on polymerase function has not been reported, mutating G500 could influence rcDNA synthesis as it is located in the RT domain of the polymerase. A previous report by Melegari et al showed that a mutant carrying F501L (located next to G500) is replication defective.[52] In any case, the enveloped HBV nucleocapsids would not be released from the cells treated with g37+g40 in the absence of HBs proteins.

Our most surprising result was the reduction of 3.5kb RNA (pgRNA) levels with g37 and g40 *in vitro*. We don't have a clear explanation for this observation, but it could be due to the impact of the mutations on viral transcription or transcript stability/degradation via nonsense-mediated RNA decay, as previously suggested for HBs RNA[20]. This warrants further studies exploring in-depth mechanistic insights. However, from the therapeutic point of view, this decrease in pgRNA is beneficial as it would contribute to reduced HBV DNA replication.

Although we have generated promising results in various models with prototypical cytosine base editor BE4, we have also demonstrated antiviral efficacy of gRNAs (g37+g40) in combination with the next generation cytosine base editors BE4-PpAPOBEC1 and CBE-T[32]. Compared with BE4, these editors have markedly reduced rates of guide-independent off-target editing in mammalian cells[31], which makes them more promising for potential therapeutic application.

Thorough evaluation of potential off-target activity is an important aspect of gene (base) editing drug discovery. Two previously published studies addressing base editing for silencing HBV genes, assessed up to 11 potential of-target sites for the investigated gRNAs[19,20]. In particular, the top three predicted off-target sites were evaluated for gRNA gS8[19], which has the same sequence as g37 used in our experiments. In this study, using rhAmpseq CRISPR analysis system[40,41] we were able to perform a much more thorough assessment: 685 potential off-target sites were evaluated for g37 and 499 off-target sites were evaluated for g40. BE4 base editor was associated with a significant number of off-target sites: 19 in case of g37 and seven in case of g40. On the contrary, BE4-PpAPOBEC1 and CBE-T had a more favorable off-target profile, while maintaining robust editing and antiviral activity in HBV-PHH cell system. A full assessment of the consequences of the detected off-target edits would be needed before making decision on the clinical use of a particular base editor with the gRNAs (g37+g40).

Taken together, our findings show that a nonviral vector can deliver cytosine base editing reagents capable of efficiently and irreversibly silencing cccDNA and integrated HBV DNA sequences in relevant *in vitro* and *in vivo* systems. These data improve our understanding of the potential of the base editing to cure HBV and contribute to our knowledge of the molecular mechanism of action by which base editing can serve as an effective antiviral.

Materials and Methods

Generation of HEK293T-lenti-HBV cell lines and transfection with the base editors (DNA format)

Two lentiviral plasmids containing partial HBV DNA sequences 2309-1622 (HBs, Pol) or 1176-2451 (X, Core) were cloned and further used for the lentiviral production. The resulting lentiviruses were transduced into HEK293T cells to generate the cell lines containing a single

partial HBV DNA sequence per cell. Hek293T-lenti-HBV cell lines were transfected with the plasmid encoding BE4 (750 ng) and a plasmid encoding gRNA (250 ng) in a 48-well plate using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. gRNA sequences are mentioned in **Tables S1 and S2.** g37 sequence has also been earlier reported by Yang et al., 2020.[19]

Hepatoma cell line culture

HepG2-NTCP and HepG2.2.15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-Glutamine (Gibco) sodium pyruvate (Gibco), 5% fetal calf serum (Fetalclone II), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) at 37°C and 5% CO2, all provided by Life Technologies (Courtaboeuf, France). 5 µg/mL puromycin (InvivoGen, Toulouse, France) and 400 µg/mL G418 (Eurobio Scientific, Les Ulis, France) were also added for HepG2-NTCP and HepG2.2.15 cells, respectively. For PLC/PRF/5 cells, Eagle's Minimum Essential Medium (ATCC, Manassas, VA) was used as a base medium with 10% heat-inactivated FBS.

HBV infection and BE4 /gRNA transfection of HepG2-NTCP

HepG2-NTCP cells were seeded at $10^5/cm^2$ in complete DMEM growth medium. From the next day onwards, cells were maintained in 2.5% DMSO (Merck-Sigma-Aldrich, Saint Quentin Fallavier, France) containing medium to enhance HBV infection.[53] After 72h, cells were infected with HBV inoculum at a multiplicity of infection of 1000 (using PEG 4%). For **Figure 2A,** the infected cells were replated at 6 dpi. Next day, these cells were transfected with BE4-encoding mRNA, and gRNA (ratio 2 :1) using Lipofectamine Messenger MAX (Life Technologies Courtaboeuf, France). In experiments using a combination of two gRNAs, mRNA: g37:g40 ratio was adjusted to 2:0.5:0.5. At 15 dpi, supernatants were collected for assessing extracellular HBV

parameters and cells were harvested for measuring intracellular parameters and DNA base editing. For 3TC treated cells as shown in **Figure S3A**, 10μM of 3TC was added at 4dpi, cells were replated at 6dpi and 3TC was maintained until 15dpi (Merck-Sigma-Aldrich, Saint Quentin Fallavier, France). For **Figure S1**, cells were replated at 2 dpi, transfection was performed next day and samples were collected at 14 dpi.

Primary Human Hepatocytes maintenance, infection, and transfection

Plated PHHs isolated from chimeric mouse liver were purchased from PhoenixBio Co.[28,29] PHHs were cultured at 350,000 cells/well concentration in a 24-well plate. Infection media was prepared using dHCGM/FBS with PEG 4% and Hepatitis B virus at MOI 500. Cells were incubated with 500 µL of infection media for 20 – 24 hours and washed next day 3 times with dHCGM/FBS media. A final media change with dHCGM/FBS was done after the 3 washes to complete infection protocol and cells were maintained at 37 degrees Celsius and 5% CO2 with media changes every 72h. Infected cells were transfected with BE4 encoding mRNA (600 ng) and gRNA (200 ng) (ratio 3:1) in each well of a 24-well plate using Lipofectamine Messenger MAX (Invitrogen) mixed with Opti-MEM media (Gibco). Cells were incubated with transfection reagents for 16-18 hours. Media (dHCGM/FBS) was changed the next day.

BE4 /gRNA transfection of HepG2.2.15 and PLC/PRF/5 cells

PLC/PRF/5 or 3TC pre-treated HepG2.2.15 cells were transfected with BE4 mRNA and gRNA (ratio 2:1). At 6th day post transfection, culture supernatants and cells were collected for detection of HBV antigens and DNA base editing, respectively.

Immunoblotting

Transfected HepG2-NTCP and HepG2.2.15 cells were washed with phosphate-buffered saline (PBS, Eurobio Scientific, Les Ulis, France) and lysed with radioimmunoprecipitation assay buffer (RIPA: 150 mM NaCl ,50 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail [Roche, Mannheim, Germany]) for 30min at 4°C followed by centrifugation at 12000g to remove cell debris. Protein concentrations were measured using BCA assay kit (Life Technologies Courtaboeuf, France). Equal amount of total protein was subjected to SDS PAGE using 4%–20%mini-PROTEAN TGX stain-Free Precast Gel or 3-8% Criterion XT Tris-Acetate (BioRad Laboratories, Marnes-la-Coquette, France). Immunodetection was done using anti-HBs (Abbott H166 mouse monoclonal), anti-Ku80 (Abcam ab119935) and anti-Cas9 (C15310258 Diagenode, Seraing, Belgium) primary antibodies followed by incubation with horseradish-peroxidase conjugated secondary antibodies. Signals were detected using Bio-Rad Clarity Western ECL and the Chemidoc XRS (Bio-Rad Laboratories, Marnes-la-Coquette, France).

Real Time PCR. Total Intracellular HBV DNA and HBV RNA.

Total cellular DNA and RNA were extracted using the Epicentre MasterPure kit (Lucigen, Middleton, WI) and the Nucleospin RNA kit (Macherey-Nagel, Hoerdt, France), respectively. Real Time PCR was performed as described earlier.[10] Briefly, total HBV DNA was quantified using Taqman PCR Pa03453406_s1(Life Technologies, Courtaboeuf, France). cccDNA amplification was performed on Exo I and II treated samples (to degrade genomic DNA and incomplete double stranded circular rcDNA intermediate species) using specific primers (For:5'CCGTGTGCACTTCGCTTCA3'; Rev:5'GCACAGCTTGGAGGCTTGA3'; Probe:5'(6FAM) CATGGAGACCACCGTGAACGCCC (BBQ). Serial dilutions of an HBV plasmid served as quantification standard. Human beta-globin (HBB) amplification (TaqMan

Assay ID: Hs00758889_s1) was performed for internal normalization of total HBV DNA or cccDNA. 3.5kb RNA quantification was done using primers For: 5' GGAGTGTGGATTCGCACTCCT3', Rev: 5'AGATTGAGATCTTCTGCGAC3'. Probe: [6FAM]AGGCAGGTCCCCTAGAAGAAGAACTCC[BBQ], and normalized to GUSB (Taqman Assay ID : Hs99999908_m1). Real time qPCRs were set up in an Applied biosystems QuantStudio 7 machine.

PHH and extracellular HBV DNA Assessment

5µl of PHH supernatant was mixed with 45µl of the buffer containing 40 µg/mL salmon sheared DNA (Invitrogen) in 10mM Tris, pH=8. Samples were boiled at 95 degrees Celsius for 15 minutes and kept on ice for qPCR preparation. HBV DNA qPCR was performed with Universal PCR Master Mix (Applied Biosystems) and DNA oligonucleotide probe ES70 (/56FAM/ccgtgtgca/ZEN/cttcgcttcacctctgc/3IABkFQ) and the primers ES72 (CCGTCTGTGCCTTCTCATCTG), and ES73 (AGTCCAAGAGTCCTCTTATGTAAGACCTT).

Detection of HBV antigens

HBsAg and HBeAg were detected in cell supernatants by ELISA using the chemiluminescence immunoassay (CLIA) kit from Autobio Diagnostic (Zheng zhou City, China) according to the manufacturer's instructions.

Southern Blot analysis

Southern Blotting was performed as described earlier using the ICE-HBV harmonized protocol [27,54] Briefly, total DNA was extracted using Hirt extraction protocol followed by treatment with Exo I/III. All samples were quantified by Qubit. Mitochondrial NADH dehydrogenase (ND2,

TaqMan Assay ID: Hs02596874_g1, Life Technologies Courtaboeuf, France) levels were quantified by qPCR and used to normalize loading. Samples were separated on 1.2% agarose gel in 1X Tris-acetate EDTA buffer at 15V. Depurination followed by denaturation and neutralization was performed in gel before transferring to a nylon membrane with 20X SSC buffer using Whatman Turbo blotter. DNA was crosslinked to the membrane by UV at 120mJ/cm2. The membrane was hybridized overnight at 55°C with DNA probes. The hybridized signal was amplified using QuantiGene Singleplex Assay kit (Life Technologies Courtaboeuf, France) and detected by Chemidoc imager.

Northern blot analysis

Total RNA was extracted using the TRI reagent (Molecular Research Center, Inc, Cincinnati, OH) protocol following recommendation from the manufacturer and quantified using Nanodrop One. 10µg of total RNA were mixed with glyoxal, denatured (50°C, 1h) and resolved 5h, 60 mV, with phosphate buffer recircularization on a 1.2% agarose gel, after which RNA integrity profile was assessed by Chemidoc imager. After RNA transfer on a Hybond N+ membrane and cross-linking (2h, 80°C), HBV RNAs were detected using DIG-labelled probes and DIG Wash and Block Buffer Set (Merck-Sigma-AldrichSigma, Saint Quentin Fallavier, France).

Next Generation Sequencing of DNA

DNA samples were sequenced by NGS (Illumina MiSeq platform) and sequencing reads were analyzed to obtain editing rates as described in the methods of Packer et al, 2022.[55]

Animal Care and Treatments

All animal care and procedures were carried out according to the relevant National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee and the

Office of Laboratory Animal Research at CRADL (Cambridge, MA). C3H male mice of 5-6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). HBV minicircle DNA was injected into C3H mice using hydrodynamic delivery as described.[38] 4 weeks after HDI mice were assessed for HBsAg and organized into the four groups for further treatment, as indicated in Figure 5. Serum was collected every 7 days from the submandibular vein and used to assess levels of HBsAg. Lipid nanoparticles were diluted in sterile 1x TBS and injected via tail vein (i.v.). Animals were euthanized 42 days post LNP injection.

Lipid nanoparticle formulations

The base editor mRNA and guide RNA were co-formulated at a weight ratio 1:1 in lipid nanoparticles. For LNPs containing a combination of two gRNAs, mRNA: g37:g40 ratio was adjusted to 1:0.5:0.5. The formulations were generated by mixing an aqueous solution of the RNA (pH of 4.0) with the four lipid components in ethanol solution: a proprietary ionizable lipid, DOPE, cholesterol, and DMG-PEG2000. The two solutions were mixed in the microfluidics device from Precision Nanosystems (Vancouver, Canada). The LNPs were dialyzed overnight against 1x Tris buffered saline at 4°C, further concentrated in 100K MWCO Amicon Ultra centrifugation tubes (Millipore Sigma, Burlington, MA) and subsequently filtered through 0.2-micron filters (Pall corporation, Port Washington, New York). Particle size was assessed using the Malvern Panalytical Zetasizer (Malvern, UK). Endotoxin was measured using Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher Scientific, Waltham, MA) following the manufacturer's protocol.

Off-target site identification with rhAmpSeq

DNA from edited and untreated cells was extracted with the PureLink Genomic DNA Mini Kit (ThermoFisher Scientific, Waltham, MA) following the manufacturer's protocol. The extracted genomic DNA was amplified with custom rhAmpSeq panels (Integrated DNA Technologies, Newark, NJ) and sequencing libraries were prepared using the rhAmpSeq Library Kit (Integrated DNA Technologies, Newark, NF). Sequencing libraries were sent to Novogene Corporation Inc. (Sacremento, CA) and were sequenced on a Novoseq S4 (Illumina Inc, San Diego, CA) to a target depth of 50,000 sequencing reads per candidate off-target site per sample.

Sequencing reads were pre-processed to trim low-quality base calls. Paired end reads were subsequently stitched to create consensus reads with adjusted base-quality scores and those stitched reads were aligned to the human reference genome. Frequencies of base calls at each position in all candidate off-target sites were calculated from the read alignments and compared across treated and untreated samples. An odds ratio quantifying the enrichment of each observed variant in the treated samples was calculated and a Fisher's exact test was used to assess statistical significance.

Data availability statement

Data that underlie the reported results will be made available upon request 3 months after publication for a period of 5 years after the publication date.

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Author Contributions

Conceptualization: EMS, MGM, EC, AK, MSP, BT, FG, FZ, GC; Formal Analysis: EMS, MGM, EC, AK, SD, DL, CYC, JRD, LAB, MSP; Funding acquisition: BT, FG, FZ, GC; Investigation: EMS, MGM, EC, AK, SD, DL, CYC, JRD, LAB, MSP, LSS, SK, LY; Methodology: EMS, MGM, EC, AK, DL, CYC, JRD, LAB, MSP, LSS, SK, LY; Supervision: BT, FG, FZ; Visualization: EMS, MGM, EC, AK, BT; Writing – original draft: EMS, MGM, EC, AK, BT, MSP; Writing – review & editing: all authors.

Declaration of interest statement

E.M.S., S.D., D.L., C.Y.C., J.R.D., L.A.B., M.S.P., G.C., L.S.S., S.K., L.Y. and F.G. are employees and shareholders of Beam therapeutics. This work was funded by Beam Therapeutics, which is developing base editing therapeutics. Beam Therapeutics has filed patent applications on this work. FZ received consulting fees from: Aligos, Antios, Assembly, Gilead, GSK; FZ and BT received research funding to INSERM from: Assembly Biosciences, Beam Therapeutics and Janssen.

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HBV, cccDNA, base editing, HBsAg, therapeutics, gene editing, lipid nanoparticles

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Figure Legends

Figure 1. cccDNA organization with the location of the two selected gRNAs g37 and g40 and gRNA/targeted sequences. The edited nucleotides and resulting amino acid changes are highlighted in red. CBE/g37 editing of the targeted cytosines C7 and/or C8 leads to the introduction of the stop codon in *HBs*; CBE/g40 editing of the cytosine C8 and/or C9 results in the stop codon in *Precore* gene.

Figure 2. Effect of CBE and gRNAs g37, g40, or their combination on HBV extracellular and intracellular parameters in HepG2-NTCP cells. (A) Experimental scheme. A protocol similar to Figure S1B was used with a few modifications. All samples were collected at 15dpi. (B-C) Extracellular HBsAg and HBeAg were measured by ELISA. (D) Total HBV DNA was quantified by qPCR from DNA extracted from cell lysates. (E) Total cellular RNA was extracted and HBV 3.5kb RNA levels were quantified by RT-qPCR. Data were normalized to PCSK9 control gRNA targeting *Proprotein convertase subtilisin/kexin type 9* (gene unrelated to HBV). (F) cccDNA level was assessed by qPCR on the DNA samples pretreated with ExoI/III, in HepG2-NTCP. (G) Level of the C>T functional editing that leads to the introduction of the stop codons in *HBs* and *Precore* genes, assessed by NGS on ExoI/III treated cccDNA samples from HepG2-NTCP, as well as PCSK9 (assessed on total DNA). Data are represented as mean ± SEM for n=6 (viral parameters) or 4 replicates (base editing).

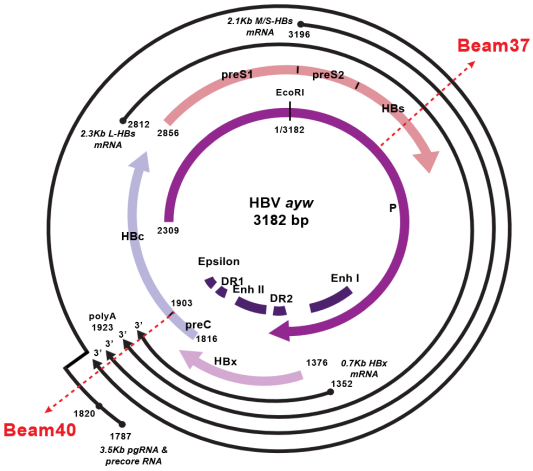
Figure 3. **Antiviral efficacy of base editing in HBV-PHH.** (A) Experimental scheme. (B) Multiplexing the two gRNAs with BE4 simultaneously reduces HBsAg, HBeAg, 3.5kb RNA, and total HBV DNA. Viral parameters assessed at the end of the experiment, day 25 post infection. BE4 with the PCSK9 gRNA was used as a control for normalizing the data. (C) HBV replication assessed by HBV DNA qPCR in primary hepatocyte (PHH) supernatant. Discontinuation of

lamivudine leads to HBV rebound, while base editing prevents viral rebound. (D) cccDNA level was assessed by qPCR on the DNA samples pretreated with ExoI/III. (E) Level of the C>T functional editing that leads to the introduction of the stop codons in *HBs* and *Precore* genes, assessed by NGS on ExoI/III treated cccDNA samples as well as PCSK9 (assessed on total DNA). Data are represented as mean ± SEM for n=3.

Figure 4. Anti-HBV efficacy of CBE & gRNAs in HBV integrated cell lines (A) Schematic representation of the protocol used for HepG2.2.15 cells. All samples were collected 6 dpt. (B-C) Extracellular HBsAg and HBeAg levels were assessed in the supernatant of the cells treated with 3TC four days before the transfection with base editing reagents (D) Level of the C>T functional editing was assessed by NGS on the purified DNA. (E) Schematic representation of the protocol used for PLC/PRF/5 cells. (F) At 6 dpt, extracellular HBsAg was measured in the supernatants of the cells transfected with g37-PLC (g37 adapted for genotype A HBs targeting site within PLC/PLF/5 cells). (G) The level of the C to T editing on HBs targeting site of g37-PLC was assessed by NGS. All ELISA data were normalized to the BE4/PCSK9 (control gRNA) condition. Error bars indicate SEM of 4 or 6 replicates.

Figure 5. LNP-mediated delivery of base editor and HBV-targeting gRNAs leads to sustained reduction of viral markers in HBV minicircle mouse model. (A) Experimental scheme. (B) Seven weeks after the injection (d0), mice injected with BE4/(g37+g40) showed > 2Log10 mean serum HBsAg reduction; 4/5 mice injected with HBV-specific LNP showed HBsAg reduction below the limit of detection. (C) HBV replication is reduced in entecavir treated mice and then rebounds when the treatment is discontinued at day 14 (positive control). Base editing treated mice showed up to 3Log10 sustained reduction in serum HBV DNA with no rebound observed. (D) Two weeks after beginning of treatment all mice treated with BE4/(g37+g40), showed HBeAg

774 below the limit of detection. (E) Total HBV DNA levels were assessed on the DNA purified from
775 mice liver at the end of the experiment. (F) Editing was assessed on the DNA purified from mice
776 liver by NGS. The data are represented as mean ± SEM, n=4 or 5 mice per group.



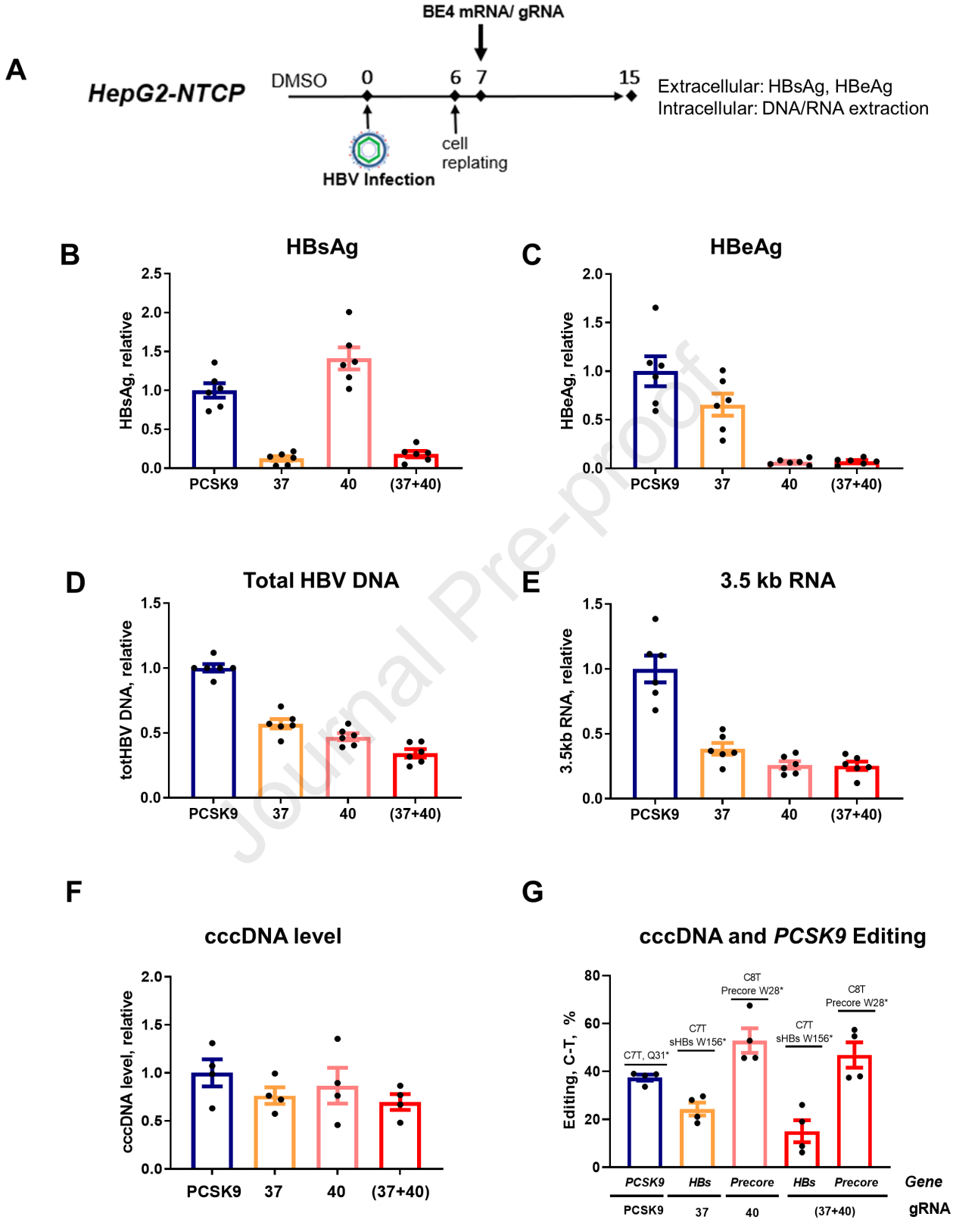
cccDNA(+) TCCCATCATCCTGGGCTTTC
gRNA37 3′-AGGGTAGTAGGACCCGAAAG-5′
8 7 6

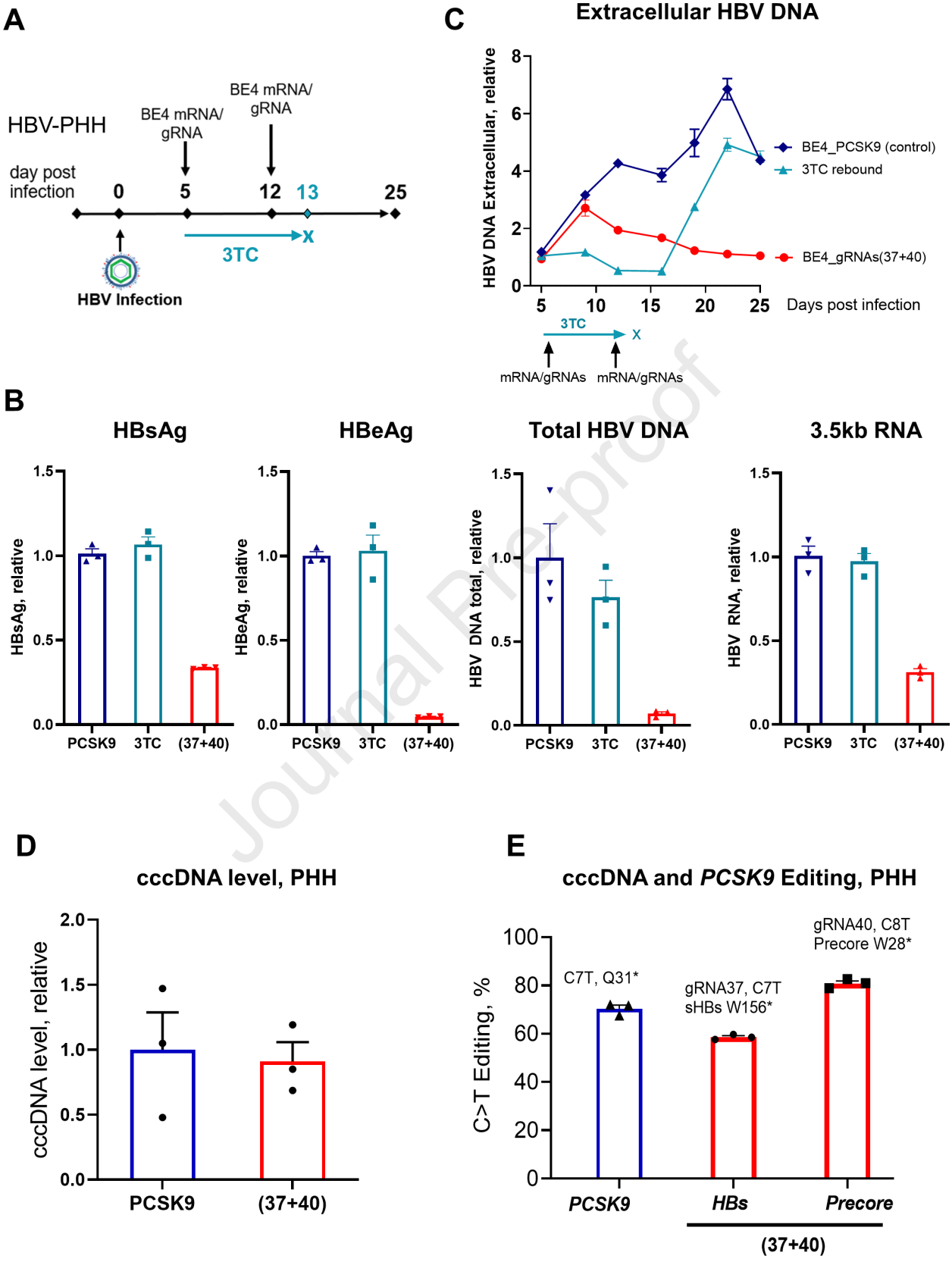
HBs gene P S S W A F
* T

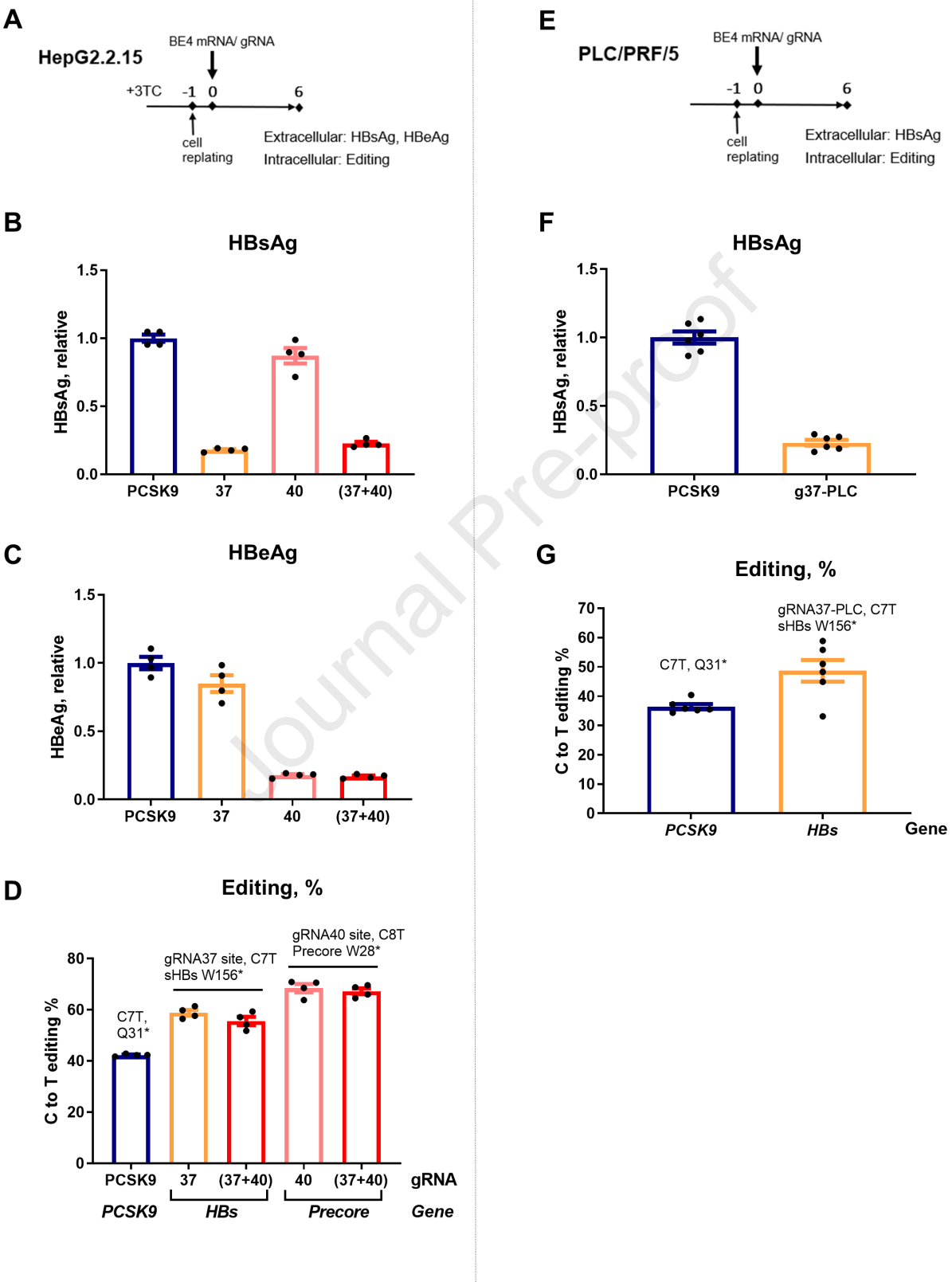
Pol gene P I I L G F
D/N

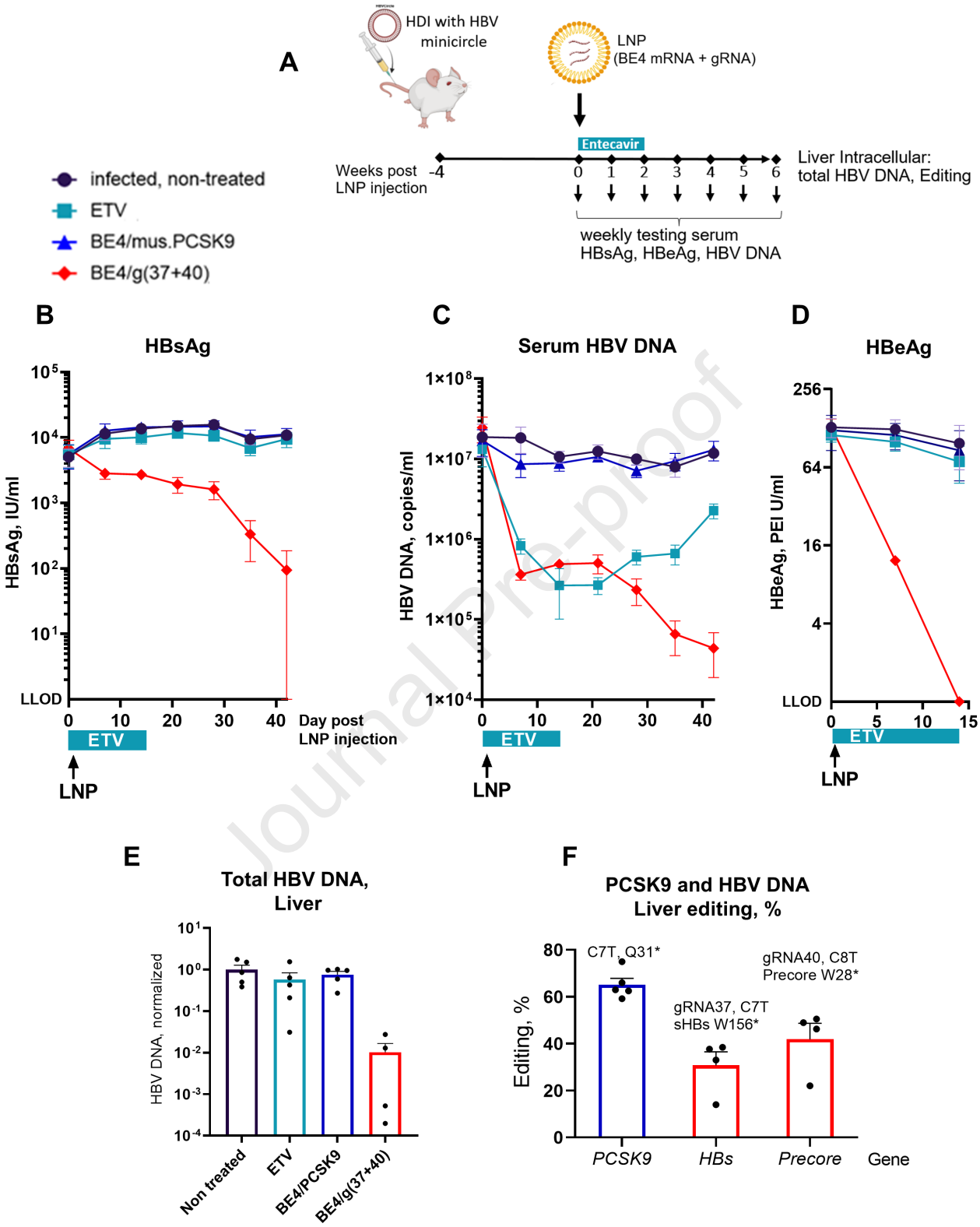
cccDNA(+) TGGGTGGCTTTGGGGCATGGAC
gRNA40 3′-ACCCACCGAAACCCCGTACC-5′
9 8 7 6

Pre(core) gene G W L W G M
* S/N /D









Zoulim, Packer and colleagues demonstrate in cell culture and in preclinical mouse model that hepatitis B virus (HBV) replication and antigen production can be durably abrogated by the use of base editing technology to introduce permanent mutations in HBV genome, opening new perspectives towards a cure for chronic HBV infection.