Adenine Base Editing enables sustained reduction of HBsAg expression and inhibition of Hepatitis B viral replication in preclinical models

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1. Unmet need in patients with chronic HBV

- Chronic Hepatitis B infection remains a global health problem (>296mln people infected worldwide, 800 000 deaths/year) (WHO, DHHS)
- HBV genome is maintained in human hepatocytes as episomal covalently closed circular DNA (cccDNA)
- cccDNA persistence in the liver is responsible for chronic HBV infection; failure to prevent HBV rebound from cccDNA is one of the key challenges to cure HBV
- HBV DNA integrates into the human genome and serves as a source of Hepatitis B Surface antigen (HBsAg) expression
- Standard antiviral medications (nucleos(t)ide analogs reverse transcriptase inhibitors) decrease viral replication, but do not provide a cure, and do not influence viral protein expression (HBsAg) from cccDNA and integrated HBV DNA

2. Adenine Base editors (ABE) convert A-T into G-C without double-stranded break (DSB)

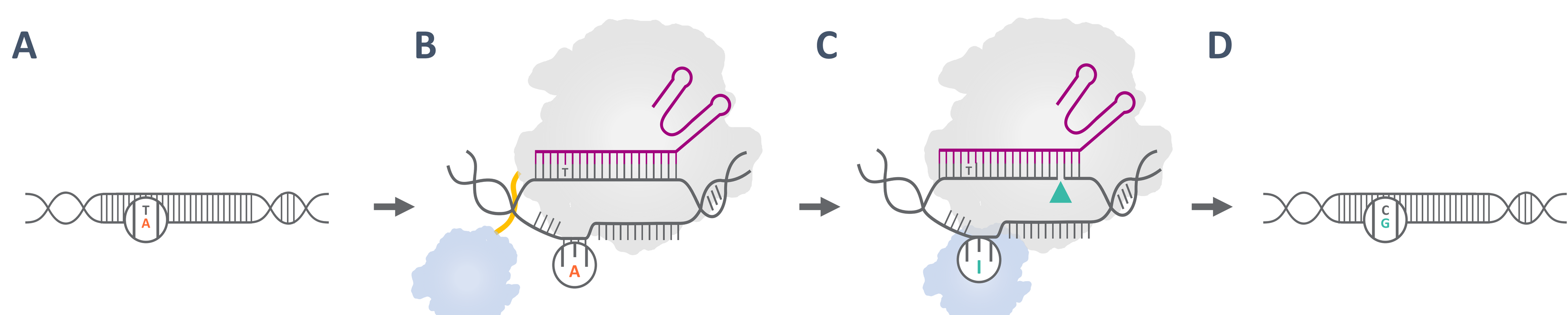
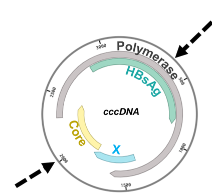


Figure 1. (A) Starting DNA sequence with the target base pair (A:T). (B) The adenine base editor (ABE) consists of an evolved TadA* deaminase (lavender) connected to partially inactivated CRISPR-Cas enzyme (grey). The base editor binds to a target sequence that is complementary to the guide-RNA (magenta) and exposes a stretch of single-stranded DNA. (C) The deaminase converts the target adenine into inosine (which is read as guanine by DNA polymerases) and the Cas enzyme nicks (▲) the opposite strand. (D) The nicked strand is repaired completing the conversion of an A:T to G:C base pair.

3. Editing strategy

- Targeting HBV genome with ABE will allow precise introduction of missense mutations silencing viral genes without generating DSB, thus minimizing risk for chromosomal rearrangements
- Base editing will address two key aspects of Chronic Hepatitis B with the same reagents:

1) Can prevent HBV rebound by introducing permanent mutations in cccDNA



2) Irreversibly silence HBsAg expression from the integrated HBV DNA without DSBs

4. Adenine Base editing reduces HBV viral parameters in infected HepG2-NTCP hepatoma cell line

- ✓ Introduce missense mutations in viral *HBV polymerase* and *HBs* genes
- ✓ Have potential to work as single gRNAs
- ✓ Introduce missense mutations in the regulatory regions of HBV genome
- ✓ Could be multiplexed with S/Pol targeting gRNAs to reduce other viral markers (HBeAg, pgRNA) and increase overall efficacy

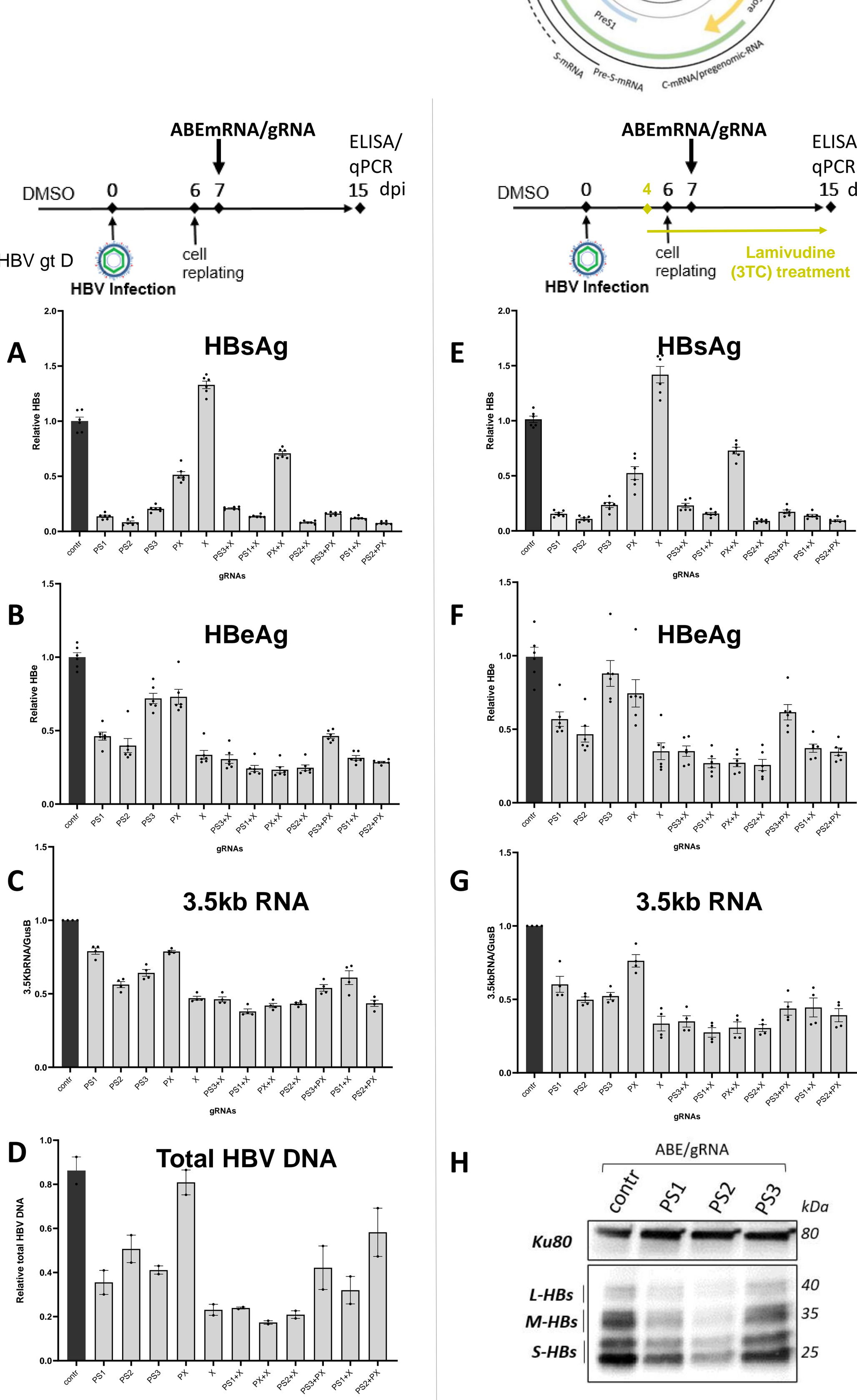


Figure 2. Adenine Base editing inhibits HBV replication and reduces viral antigens level in infected HepG2-NTCP cells.

Extracellular HBsAg and HBeAg were measured by ELISA. Total HBV DNA was quantified by qPCR from DNA extracted from cell lysates. Total cellular RNA was extracted and HBV 3.5kb RNA levels were quantified by RT-qPCR. Data were normalized to ABE&control gRNA targeting *ALAS1* gene unrelated to HBV.

(A, D) ABE with gRNAs targeting overlapping *S* and *Polymerase* genes enable reduction of HBsAg and HBeAg DNA;

(B-D) gRNA_X enables robust reduction of HBeAg, HBV DNA, and viral pgRNA

(A-D) Multiplexing S/Pol and X targeting gRNAs allows for strong reduction across all viral parameters.

(E-G) Combination with 3TC showed that the inhibition effects are maintained in the context of reduced replicative intermediates, suggesting MoA via direct cccDNA base editing

(H) Western blot shows an intracellular decrease in the three HBs isoforms (L, M, and S) upon treatment with Abe&HBV gRNAs, with the most profound effect achieved for ABE/gRNA_PS2.

5. Base Editing reduces HBsAg expression in PLC/PRF/5 cells from naturally integrated HBV DNA

- PLC/PRF/5 is hepatoma cell line with naturally integrated HBV DNA derived from patient (HBV genotype A (Ishii et al, 2020).

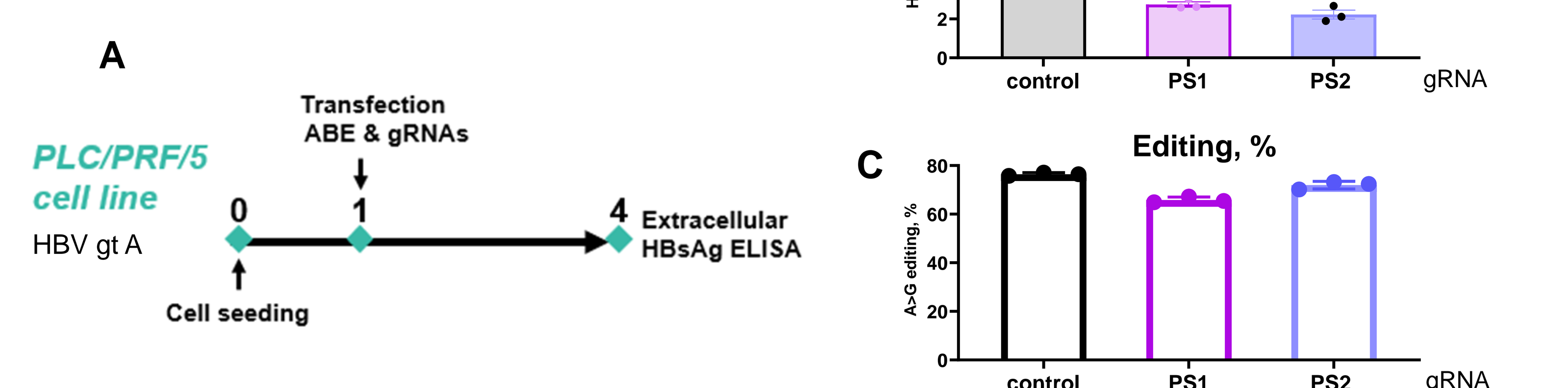
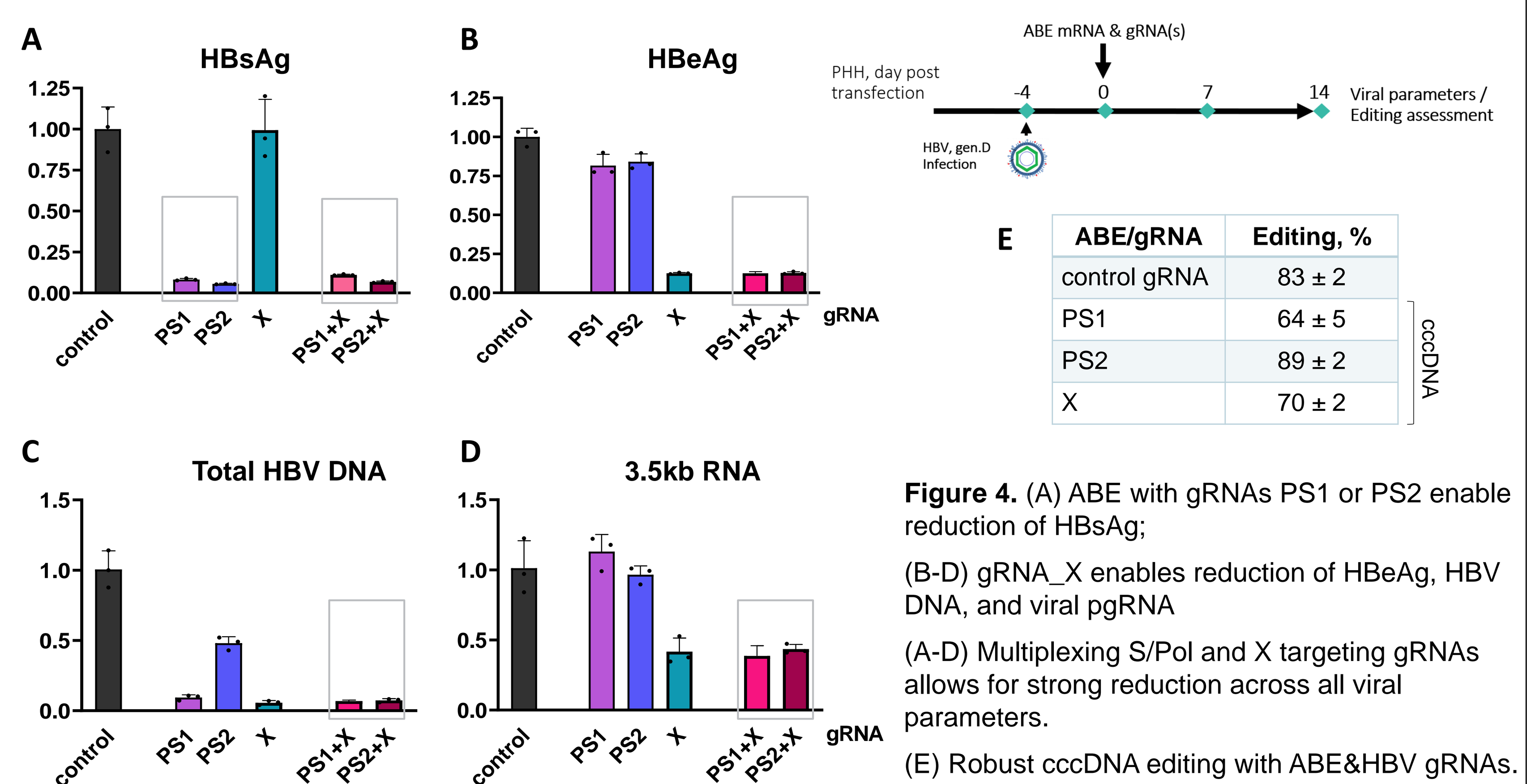


Figure 3. (A) Experimental protocol;
(B) Extracellular HBsAg levels were determined by ELISA at the 4th day post-transfection with ABE and gRNAs PS1 or PS2. (C) ~60-75% editing of *HBs* gene was achieved to enable robust reduction of HBsAg.

6. ABE reduces HBV viral parameters, including HBsAg level, in primary human hepatocytes (PHH)



ABE/gRNA	Editing, %
control gRNA	83 ± 2
PS1	64 ± 5
PS2	89 ± 2
X	70 ± 2

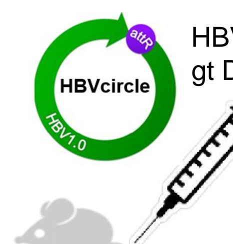
Figure 4. (A) ABE with gRNAs PS1 or PS2 enable reduction of HBsAg;

(B-D) gRNA_X enables reduction of HBeAg, HBV DNA, and viral pgRNA

(A-D) Multiplexing S/Pol and X targeting gRNAs allows for strong reduction across all viral parameters.

(E) Robust cccDNA editing with ABE&HBV gRNAs.

7. In vivo PoC in HBVcircle mouse model: ABE enables loss of HBsAg and sustained 98% reduction in HBV DNA



- **HBVcircle mouse model** supports durable production of HBV-like viral particles and HBV antigen expression in immunocompetent mice (Yan et al, 2017)
- 4 weeks after hydrodynamic injection with cccDNA-like minicircle plasmid, mice received one dose of base editing reagents (mRNA&gRNA formulated in a lipid nanoparticle (LNP)
- Entecavir (SOC) treated mice received antiviral at 0.03mg/kg orally for two weeks, then the treatment was discontinued to reveal viral rebound.

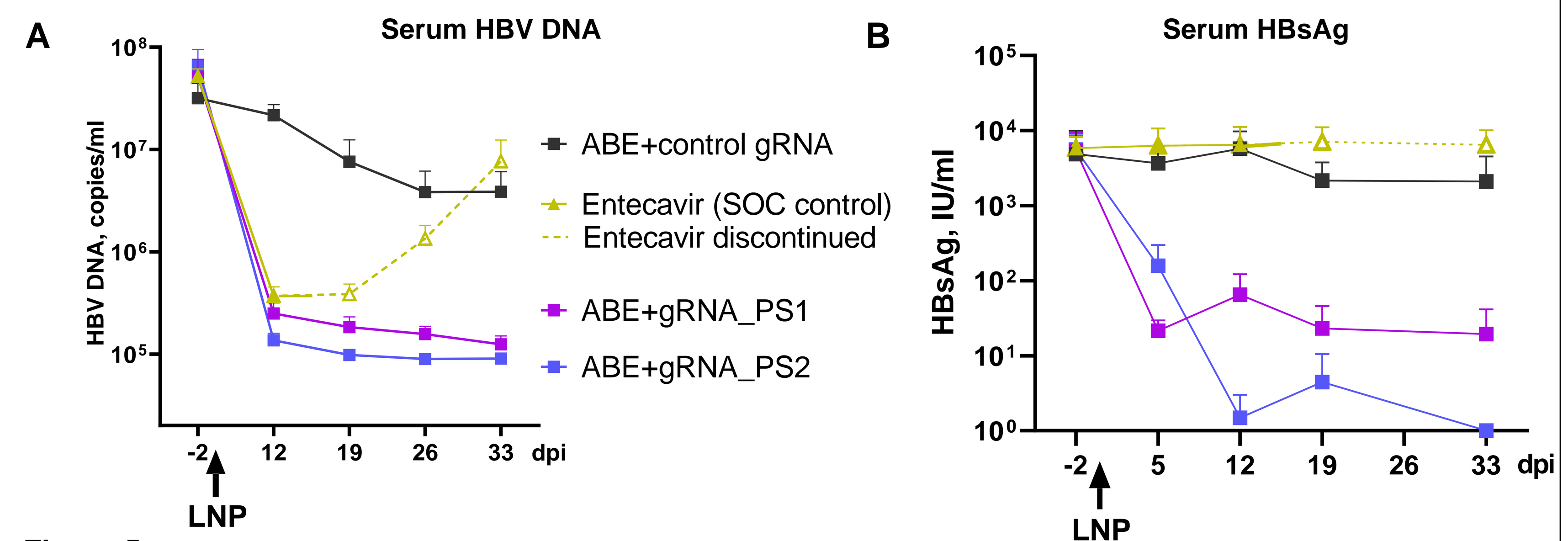
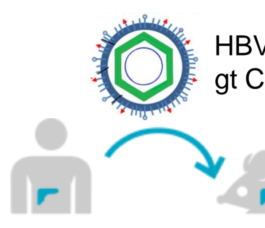
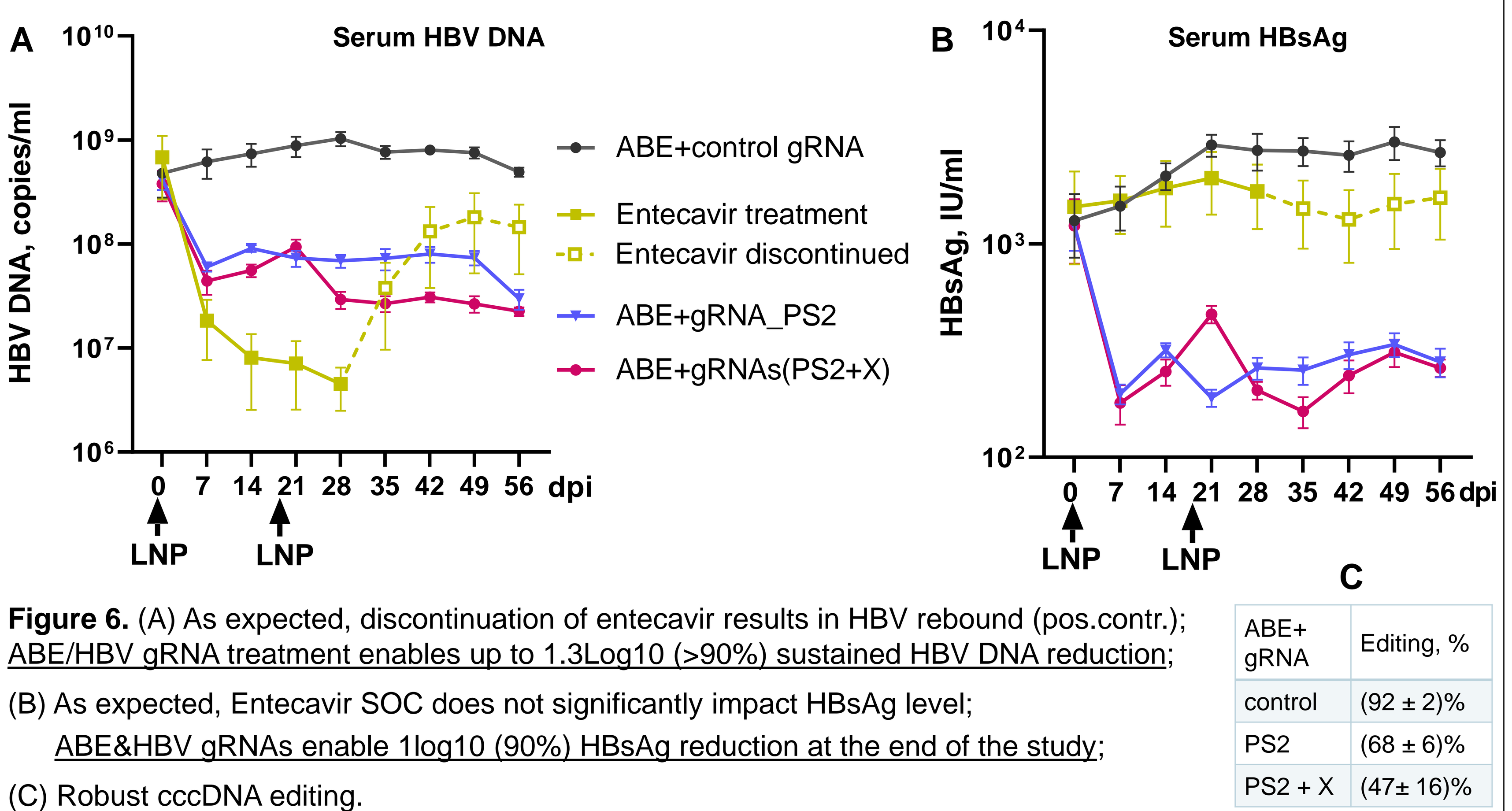


Figure 5.
(A) HBV replication is reduced in entecavir treated mice, and then rebounds as soon as the treatment is discontinued (positive control);
98% sustained reduction in serum HBV DNA in ABE&HBV gRNAs treated groups; no HBV rebound;
(B) As expected, entecavir does not influence HBsAg level;
3-4 log sustained HBsAg reduction in ABE&HBV gRNAs treated groups > 4 weeks after a single LNP injection.

8. In vivo PoC in humanized mice: ABE enables sustained 90% reduction in serum HBsAg and HBV DNA



- **HBV-PXB Humanized mice**, > 80% of the liver repopulated with human hepatocytes, immunocompromised background (PhoenixBio)
- Infection with live virus; human cells enable reinfection and cccDNA recycling
- Mice received two doses of LNP/(ABE+gRNA)
- Entecavir treated mice received antiviral for 28 days, the the treatment was stopped



ABE+ gRNA	Editing, %
control	(92 ± 2)%
PS2	(68 ± 6)%
PS2 + X	(47± 16)%

Figure 6. (A) As expected, discontinuation of entecavir results in HBV rebound (pos.contr.); ABE/HBV gRNA treatment enables up to 1.3Log10 (>90%) HBV DNA reduction;
(B) As expected, Entecavir SOC does not significantly impact HBsAg level;
ABE&HBV gRNAs enable 1log10 (90%) HBsAg reduction at the end of the study;
(C) Robust cccDNA editing.

9. Conclusions

- Adenine Base Editing enables sustained reduction of the two major HBV markers, HBsAg and HBV DNA, in relevant *in vitro* and *in vivo* models
  - ABE base editing reduces HBsAg, HBeAg, HBV DNA, and 3.5kb RNA in HepG2-NTCP cells and primary human hepatocytes;
  - Combining base editing reagents with standard antiviral lamivudine does not compromise anti-HBV effect;
  - Base editing strongly reduces HBsAg expression from integrated HBV sequences;
  - Complete loss of HBsAg (3-4 log10) in HBV minicircle mouse model;
  - 1log10 HBsAg reduction in HBV humanized mice.
- Potential to use single gRNA to inhibit HBsAg/HBV replication or multiplexing gRNAs to reduce other viral markers (HBeAg, 3.5kb viral RNA)
- Potential to enable sustained effect with a single dose treatment

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