The gRNA-miRNA-gRNA ternary cassette combining CRISPR/Cas9 with RNAi approach strongly inhibits hepatitis B virus replication

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Supplementary materials

Table S1. Oligo-nucleotides sequences for constructing gRNA-miR-HBV-gRNA cassette

Name	Sequences
Pri-miR-31(22+21)/ miR-HBV	Top: 5'-TCGAGctgtaactcggaactggagaggGGTGAAGCGAAGTGCAC ACGGgttgaactgggaacgACGTGTGCACATCGATTCACGGCtttcctgtctgaca gcagcttG-3' Bottom: 5'-GATCCaagctgctgtcagacaggaaaGCCGTGAATCGATGTG CACACGTcgttcccagttcaacCCGTGTGCACTTCGCTTCACCcctctccagttcc gagttacagC-3'
Pri-miR-31(30+31)/ miR-HBV	Top: 5'-TCGAGtattgctcctgtaactcggaactggagaggGGTGAAGCGAAGT GCACACGGgttgaactgggaacgACGTGTGCACATCGATTCACGGCtttcctg tctgacagcagcttggctacctccG-3' Bottom: 5'-GATCCggaggtagccaagctgctgtcagacaggaaaGCCGTGAA TCGATGTGCACACGTcgttcccagttcaacCCGTGTGCACTTCGCTTCACC cctctccagttccgagttacaggagcaataC-3'
gRNA3	Top:5'-CACCGCAAGCCTCCAAGCTGTGCCT-3' Bottom:5'-AAACAGGCACAGCTTGGAGGCTTGC-3'
gRNA4	Top: 5'-CACCGCGAGGGAGTTCTTCTTCTAG-3'

	Bottom: 5’-AAACCTAGAAGAAGAACTCCCTCGC-3’

Table S2. The synthesized gene sequences for constructing gRNA-miR-HBV-gRNA ternary cassette and gRNA-gRNA binary cassette

Name	Sequences
Pri-miR-31(38+40)/ miR-HBV	5’-agaCTCGAGagggatggtattgctcctgtaactcggaactggagaggGGTG AAGCGAAGTGCACACGGgttgaactgggaacgACGTGTGCACATCG ATTCACGGCtttcctgtctgacagcagcttggctacctccgtcctgttcGGATCCgag-3’
Pri-miR-31(38+40)/ miR-HBVm	5’-agaCTCGAGagggatggtattgctcctgtaactcggaactggagaggGGTG AAGCGAATGGCATAGCGgttgaactgggaacgAGCTATGCCACATC GATTCACGGCtttcctgtctgacagcagcttggctacctccgtcctgttcGGATCCgag-3’
Pri-miR-31(51+51)/ miR-HBV-gRNA1	5’-agaCTCGAGcataacaacgaagagggatggtattgctcctgtaactcggaact ggagaggGGTGAAGCGAAGTGCACACGGgttgaactgggaacgACGTG TGCACATCGATTCACGGCtttcctgtctgacagcagcttggctacctccgtcctgttc ctccttgtcttGGATCCgagCCTGCTGGTGGCTCCAGTTCGTTTTAGA GCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAA CTTGAAAAAGTGGCACCGAGTCGGTGCttttttaccAAGCTTgca-3’
Pri-miR-31(51+51)/ miR-HBV-gRNA2	5’-agaCTCGAGcataacaacgaagagggatggtattgctcctgtaactcggaact ggagaggGGTGAAGCGAAGTGCACACGGgttgaactgggaacgACGTG TGCACATCGATTCACGGCtttcctgtctgacagcagcttggctacctccgtcctgttc ctccttgtcttGGATCCgagGGTTGCGTCAGCAAACACTGTTTTAGA GCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAA CTTGAAAAAGTGGCACCGAGTCGGTGCttttttaccAAGCTTgca-3’
U6-gRNA2	5’-agaCTCGAGttttttgagggcctatttcccatgattccttcatatttgcatatacgata caaggctgttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtg acgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgctt accgtaacttgaaagtatttcgatttcttggctttatatatcttGTGGAAAGGACGAAAC ACCGGTTGCGTCAGCAAACACTGTTTTAGAGCTAGAAATAG

	CAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT GGCACCGAGTCGGTGCtttttttaccAAGCTTgca-3’

Table S3. Target sequences of the HBV-specific gRNAs in HBV genome

gRNA No.	Sequence ($N_{19\text{-}20}$*NGG*, 5’-3’)	Genotype
1	CCTGCTGGTGGCTCCAGTTC	A/B/C/D
2	AGTGTTTGCTGACGCAACC	A/B/C/D
3	CAAGCCTCCAAGCTGTGCCT	A/B/C/D
4	CTAGAAGAAGAACTCCCTCG	A/B/C/D

Table S4. The full DNA sequences of all gRNAa-miR-HBV-gRNAb cassettes

Name	Sequences
3-H51-2	5’-GCAAGCCTCCAAGCTGTGCCTGTTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG AGTCGGTGC**CTCGAG**cataacaacgaagagggatggtattgctcctgtaactcggaactgg agaggGGTGAAGCGAAGTGCACACGGgttgaactgggaacgACGTGTGCA CATCGATTCACGGCtttcctgtctgacagcagcttggctacctccgtcctgttcctccttgtcttG **GATCC**gagGGTTGCGTCAGCAAACACTGTTTTAGAGCTAGAAATA GCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG CACCGAGTCGGTGCtttttttaccAAGCTT-3’
3-H38-2	5’-GCAAGCCTCCAAGCTGTGCCTGTTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG AGTCGGTGC**CTCGAG**agggatggtattgctcctgtaactcggaactggagaggGGTGA AGCGAAGTGCACACGGgttgaactgggaacgACGTGTGCACATCGATTC ACGGCtttcctgtctgacagcagcttggctacctccgtcctgttc**GGATCC**gagGGTTGCG TCAGCAAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCt tttttaccAAGCTT-3’

3-M38-2	5’-GCAAGCCTCCAAGCTGTGCCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC**CTCGAGG**agggatggtattgctcctgtaactcggaactggagaggGGTGAAGCGAATGGCATAGCGgttgaactgggaacgAGCTATGCCAATCGATTCACGGCctttcctgtctgacagcagcttggctacctccgtcctgttc**GGATCC**gagGGTTGCGTCAGCAAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCtttttaccAAGCTT-3’
3-H30-2	5’-GCAAGCCTCCAAGCTGTGCCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC**CTCGAG**tattgctcctgtaactcggaactggagaggGGTGAAGCGAAGTGCACACGGgttgaactgggaacgACGTGTGCACATCGATTCACGGCttcctgtctgacagcagcttggctacctcc**GGATCC**gagGGTTGCGTCAGCAAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCtttttaccAAGCTT-3’
3-H22-2	5’-GCAAGCCTCCAAGCTGTGCCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC**CTCGAG**ctgtaactcggaactggagaggGGTGAAGCGAAGTGCACACGGgttgaactgggaacgACGTGTGCACATCGATTCACGGCttcctgtctgacagcagctt**GGATCC**gagGGTTGCGTCAGCAAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCtttttaccAAGCTT-3’
4-H38-1	5’-GCGAGGGAGTTCTTCTTCTAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC**CTCGAG**agggatggtattgctcctgtaactcggaactggagaggGGTGAAGCGAAGTGCACACGGgttgaactgggaacgACGTGTGCACATCGATTCACGGCttcctgtctgacagcagcttggctacctccgtcctgttc**GGATCC**gagCCTGCTGGTGGCTCCAGTTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCtttttaccAAGCTT-3’

The red letters were the sequences of gRNA, the sequences of HBV specific short guide RNA were highlighted in green for gRNAa and in yellow for gRNAb; The blue letters were the sequences of pri-miR31 and anti-HBV pri-miR-31 mimic, the sequences of miRNA were highlighted in dark blue; The sequences of XhoI and HindIII restriction endonucleases cutting sites were shown in bold fonts.

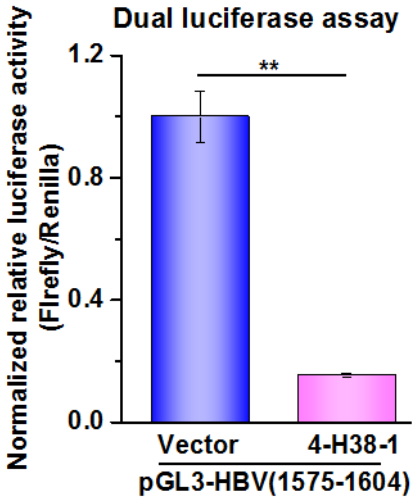

Figure S1. A dual-luciferase assay was conducted to analyze the level of miR-HBV produced by 4-H38-1 cassette. pGL3-HBV (1575-1604), PRL-TK and the expression plasmid containing 4-H38-1 cassette were co-transfected into HuH7 cells, and the dual-luciferase assay was performed. Data was shown as mean±SD of 5 independent experiments.

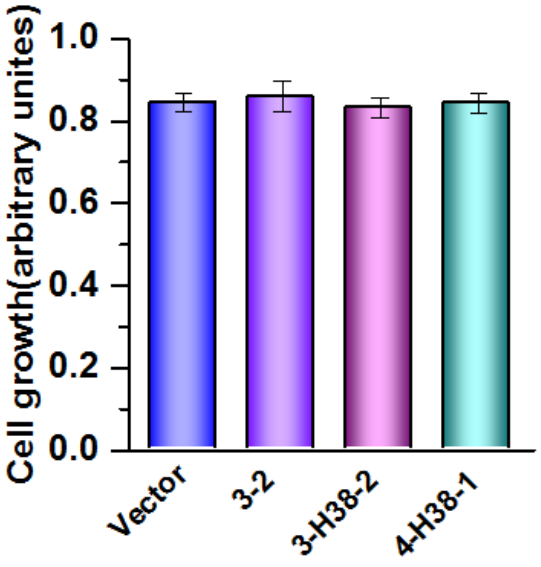

Figure S2. The cytotoxicity of 3-2 binary and gRNA-miR-HBV-gRNA ternary cassettes was examined using an MTT assay. Data was shown as mean±s.e.m. of 5 independent experiments.

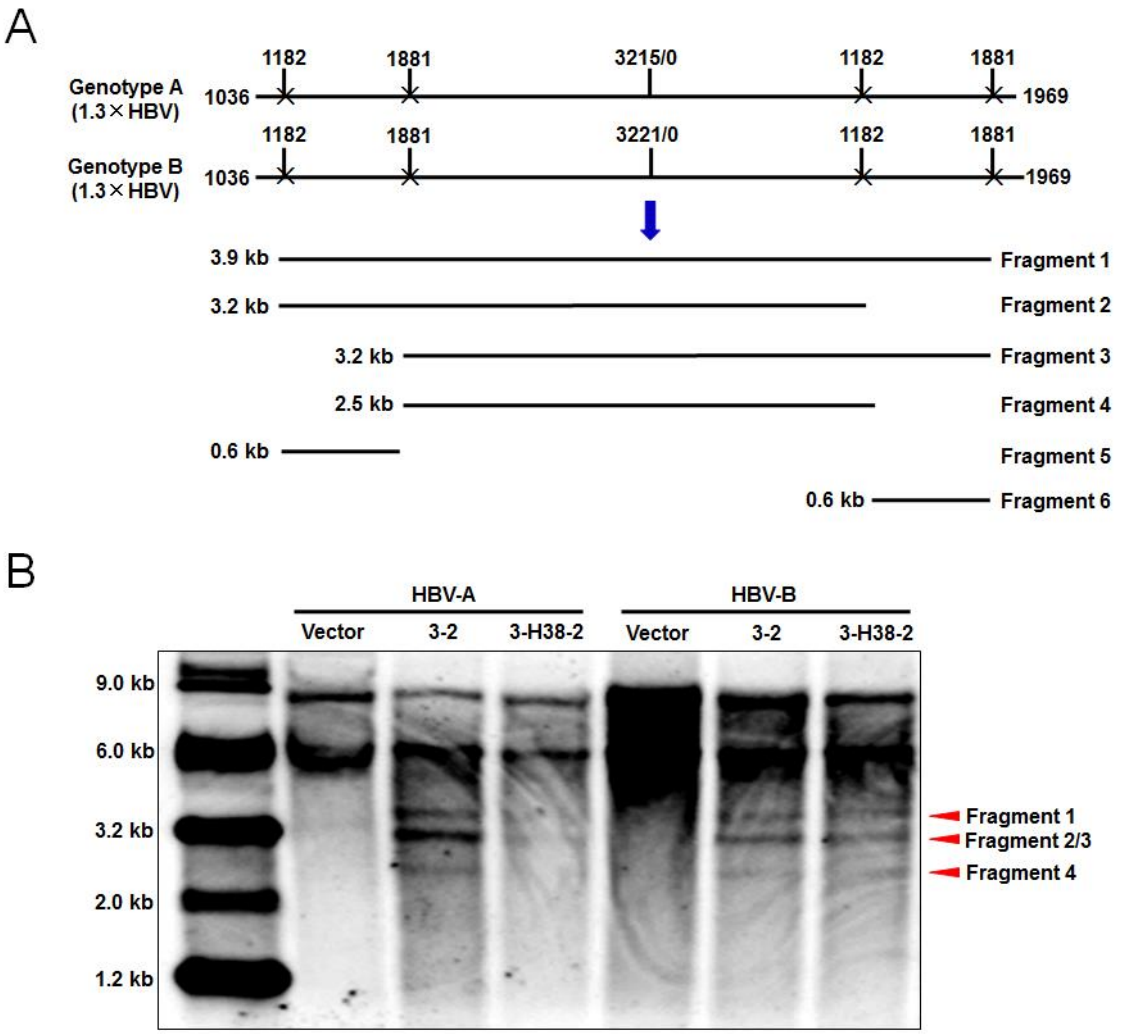

Figure S3. The HBV-specific gRNAs-mediated destructions of HBV genome were detected by Southern Blot. (A) Schematic illustration of Cas9/gRNA-mediated destruction of HBV genome (1.3×HBV expression plasmid). (B) The Cas9/gRNA-mediated destructions of HBV genome (1.3×HBV expression plasmid) were detected by Southern Blot. Vector group is the PX458 plasmid (the backbone of 3-2 and 3-H38-2 expression plasmid) was contransfected with pGEM-HBV1.3A or pGEM-HBV1.3B into HuH7 cells.