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Improved antiviral efficacy using TALEN-mediated homology directed recombination to introduce artificial primary miRNAs into DNA of hepatitis B virus

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ABSTRACT

Chronic infection with hepatitis B virus (HBV) remains an important global health problem. Currently licensed therapies have modest curative efficacy, which is as a result of their transient effects and limited action on the viral replication intermediate comprising covalently closed circular DNA (cccDNA). Gene editing with artificial HBV-specific endonucleases and use of artificial activators of the RNA interference pathway have shown anti-HBV therapeutic promise. Although results from these gene therapies are encouraging, maximizing durable antiviral effects is important. To address this goal, a strategy that entails combining gene editing with homology-directed DNA recombination (HDR), to introduce HBV-silencing artificial primary microRNAs (pri-miRs) into HBV DNA targets, is reported here. Previously described transcription activator-like effector nucleases (TALENs) that target the *core* and *surface* sequences of HBV were used to introduce double stranded breaks in the viral DNA. Simultaneous administration of donor sequences encoding artificial promoterless anti-HBV pri-miRs, with flanking arms that were homologous to sequences adjoining the TALENs' targets, augmented antiviral efficacy. Analysis showed targeted integration and the length of the flanking homologous arms of donor DNA had a minimal effect on antiviral efficiency. These results support the notion that gene editing and silencing may be combined to effect improved inhibition of HBV gene expression.

1. Introduction

Hepatitis B virus (HBV) infection is hyper-endemic to Sub-Saharan Africa and East Asia, and remains a global health priority [1]. It is estimated that 240 million people are chronic carriers of the virus and these individuals are at high risk for complicating cirrhosis and hepatocellular carcinoma. Currently licensed therapies for HBV infection include nucleoside analogs, nucleotide analogs and derivatives of interferon alpha (IFN-α) [2,3]. Although these therapies diminish replication of HBV, treatment interruption usually results in relapse with proliferation of the virus. The main reason for the modest cure rate of licensed regimens is that efficacy of the drugs is short-lived and the transcriptional template of HBV, comprising covalently closed circular DNA (cccDNA), is unaffected by available drugs [4,5]. Licensed vaccination against the virus usually prevents HBV infection, but has little or no therapeutic benefit. Developing improved methods of countering HBV thus remains an important priority and inactivating cccDNA is essential to cure the infection.

Employing strategies of gene therapy to inactivate HBV replication permanently has recently shown promise (reviewed in Ref. [6]). Gene editing and gene silencing inactivate HBV and both approaches have been shown to be effective without causing toxicity and other unintended off-target effects. Transcription activator-like effector nucleases (TALENs) [7,8] and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) nucleases [9–12] have been engineered to target HBV and are capable of introducing mutations at specific

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sites of viral DNA. Repeated digestion of target viral DNA to form double strand breaks (DSBs) eventually results in introduction of site-specific mutations. Error-prone non-homologous end joining (NHEJ), required to repair cleaved DNA, eventually leads to mutation of the cccDNA. In addition to gene editing, activation of RNA interference (RNAi) may be used to suppress HBV (reviewed in Ref. [13]). Both synthetic and expressed RNAi activators have good efficacy against HBV. Also, expressed HBV-targeting RNAi activators that generate multiple guides may improve antiviral efficacy and prevent the emergence of escape mutants [14].

In dividing cells, such as are often found in the livers of patients chronically infected with HBV, homology directed recombination (HDR) occurs efficiently (reviewed in Refs. [15,16]). HDR is based on producing DSBs at specific target sites and simultaneous introduction of 'repairing' donor DNA. The donor DNA typically has flanking arms with sequences that are complementary to those surrounding the DSBs. To augment efficacy of gene therapy against HBV, we investigated the utility of combining gene editing with use of HDR. The strategy was employed to introduce tricistronic artificial anti-HBV primary microRNAs (pri-miRs) [14] into viral target DNA. We used previously described TALENs that target *core* and *surface* sequences of the HBV genome [7]. To integrate *X*-targeting pri-miRs into viral DNA, linear donor sequences with flanking homologous regions were also introduced into liver-derived cells. Augmented efficacy and intended target site integration were observed.

2. Materials and methods

2.1. Plasmids and donor sequences

HBV-targeting TALENs [7] and artificial pri-miRs [14] have previously been described. Plasmids that included left and right homologous arms (HAs), each comprising 300 bp of HBV sequences upstream and downstream of the C-TALEN and S-TALEN target sites, were generated using standard conditions of PCR and plasmid cloning. The Kapa HiFi Mix (Kapa Biosystems, MA, USA) was used to generate DNA and primer sets for amplification are described in Supplementary Table 1. Briefly, a bipartite PCR approach was used to amplify the 300 bp left HA and right HA of the predicted TALEN cleavage sites. Primers were designed to include unique restriction sites, complementary sequences and disrupting stop codons that were in frame with the viral ORFs. Amplicons were purified and a second primer-free annealing PCR was carried out to join the left and right HAs. The 5′ ends of the left HA reverse primers and right HA forward primers had unique 20 bp complementary sequences to facilitate annealing and extension of the HAs. Complete surface and core donor templates were inserted into pTZ57R/T using the InsTAclone PCR cloning kit (Thermo Scientific, MA, USA) to generate pTZ-C300 and pTZ-S300. The trimeric pri-miR-31/5/8/9 sequence was excised from pCI-pri-miR-31/5/8/9 [14] using *Xba*I and *Nhe*I and inserted into pTZ-C300 and pTZ-S300 at the unique *Nhe*I site to generate pTZ-C300-pri-miR-31/5/8/9 and pTZ-S300-pri-miR-31/5/8/9. Plasmids with and without the pri-miR-31/5/8/9 element were used as templates to amplify linear donors with left and right HAs, each of 50 bp, 100 bp, 150 bp and 300 bp. Prior to transfection, donor template strands were purified with the Gene JET PCR Purification Kit (Thermo Scientific, MA, USA).

2.2. Transfection of liver-derived cells and HBV knockdown analysis by ELISA

Liver-derived Huh7 and HepG2.2.15 [17] cell lines were used to assess antiviral efficacy and TALEN-mediated pri-miR integration in culture. Linear HDR donor templates, together with plasmids expressing TALENs and the HBV replication-competent plasmid, pCH-9/3091 [18,19], were used to co-transfect Huh7 cells transiently. Cells were cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 10% FCS (Gibco™, Thermo Scientific, MA, USA), penicillin (100,000 U/ml), streptomycin (100,000 μg/ml), and maintained in a humidified incubator at 37 °C and 5% CO_2.

One day prior to transfection, Huh7 cells were seeded in 12-well plates at a density of 120,000 cells per well. Cells were transfected using polyethylenimine with the following combinations of plasmids: 200 ng pCH-9/3091, 100 ng pCMV-GFP, 100 ng linear HDR donor sequences of varying lengths, 800 ng of each plasmid expressing the left and right subunits of the TALENs, or 1600 ng of pUC118. Fluorescence microscopy was used to detect GFP expression and confirm equivalent transfection efficiencies. HBsAg concentrations were measured using the Monolisa™ HBs Ag ULTRA kit (Bio-Rad, CA, USA).

One day prior to transfection, HepG2.2.15 cells were seeded in 6-well plates at a density of 140,000 cells per well. Two hours before transfection, culture medium was collected to measure baseline HBsAg concentrations. Cells were transfected using polyethylenimine with the following combinations of plasmids: 150 ng pCMV-GFP, 250 ng HDR donor templates and 2.3 μg of each plasmid expressing the left and right subunits of the TALENs, or 4.6 μg of pUC118. Cells were maintained under mildly hypothermic conditions (30 °C and 5% CO_2) [4]. Growth medium was replaced and HBsAg concentrations were measured on days 3, 5 and 7. On day 7, cells were harvested and a 1:5 dilution reseeded before repeat transfection.

2.3. Evaluation of targeted integration

A PCR-based assay was used to confirm that targeted integration occurred at the intended site of the viral DNA. Primers complementary to sequences within the HBV *core* or *surface* sequences, but outside of the homology arms of the donor DNA were used to amplify mutated and wild type viral sequences (Supplementary Table 1). Corroboration of the intended HDR was carried out by sequencing the amplicons (Inqaba Biotechnologies, Pretoria, South Africa). To measure targeted indel formation, HBV DNA was extracted from HepG2.2.15 cells and subjected to analysis using a T7E1 assay as has been described [4].

2.4. Statistical analysis

Data are represented as the mean ± standard error of the mean. Statistical difference was considered significant when $P < 0.05$ and was obtained using the nonparametric Student's *t*-test with the GraphPad Software (GraphPad Software, Inc., CA, USA).

3. Results

3.1. Using TALENs to introduce artificial anti-HBV pri-miRs into viral DNA

Dimeric engineered nucleases were generated to cleave sequences within the *core* and *surface* open reading frames of HBV [7], and are termed C-TALEN and S-TALEN respectively (Fig. 1A). The left and right subunits that constitute the TALENs bind two sequences of 19 nucleotides, each with a T residue at the 5′ end. *Fok*I nuclease domains were positioned at the C-terminal end of each of the TALEs and the dimers designed to be oriented in a head-to-head arrangement with an intervening spacer of 13 bp. The complete TALEN subunits each also included a nuclear localization signal (NLS). Trimeric pri-miRs generate silencing sequences with cognates in the *X* ORF (Fig. 1A) and the artificial gene silencers have a

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