



RESEARCH ARTICLE

Cloning of human *ABCB11* gene in *E. coli* required the removal of an intragenic Pribnow-Schaller Box before it's Insertion into genomic safe harbor AAVS1 site using CRISPR-Cas9

[version 1; peer review: 2 approved]

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V1 First published: 23 Dec 2020, 9:1498
<https://doi.org/10.12688/f1000research.26659.1>

Latest published: 23 Dec 2020, 9:1498
<https://doi.org/10.12688/f1000research.26659.1>

Abstract

Background: Genomic safe harbors are sites in the genome which are safe for gene insertion such that the inserted gene will function properly, and the disruption of the genomic location doesn't cause any foreseeable risk to the host. The AAVS1 site is the genetic location which is disrupted upon integration of adeno associated virus (AAV) and is considered a 'safe-harbor' in human genome because about one-third of humans are infected with AAV and so far there is no apodictic evidence that AAV is pathogenic or disruption of AAVS1 causes any disease in man. Therefore, we chose to target the AAVS1 site for the insertion of *ABCB11*, a bile acid transporter which is defective in progressive familial intra hepatic cholestasis type-2 (PFIC-2), a lethal disease of children where cytotoxic bile salts accumulate inside hepatocytes killing them and eventually the patient.

Methods: We used the CRISPR Cas9 a genome editing system to insert the *ABCB11* gene at AAVS1 site in human cell-lines.

Results: We found that human *ABCB11* sequence has a "Pribnow-Schaller Box" which allows its expression in bacteria and expression of *ABCB11* protein which is toxic to *E. coli*; the removal of this was required for successful cloning. We inserted *ABCB11* at AAVS1 site in HEK 293T using CRISPR-Cas9 tool. We also found that the *ABCB11* protein has similarity with *E. coli* endotoxin (lipid A) transporter MsbA.

Conclusions: We inserted *ABCB11* at AAVS1 site using CRISPR-Cas9; however, the frequency of homologous recombination was very low for this approach to be successful *in vivo*.

Open Peer Review

Reviewer Status  

	Invited Reviewers	
	1	2
version 1		
23 Dec 2020	report	report

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2. **Nagendra Chaturvedi**, University of Nebraska Medical Center, Omaha, USA

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Keywords

Progressive Familial Intra Hepatic Cholestasis Type-2, CRISPR-Cas9, AAVS1 site, human ABCB11/BSEP, MsbA, E. Coli, Endotoxin, Lipid A transporter, Pribnow-Schaller Box, cloning

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Author roles: **Vats N:** Investigation; **Sanal MG:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Venugopal SK:** Writing – Review & Editing; **Taneja P:** Writing – Review & Editing; **Sarin SK:** Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: The corresponding author (Sanal MG) acknowledges the Science and Engineering Research Board (grant #ECR/2015/000275) and Department of Biotechnology, Ministry of Science and Technology, India (grant #BT/PR15116/MED/31/334/2016) Government of India for a limited financial support for about 18 months. *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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How to cite this article: Vats N, Sanal MG, Venugopal SK *et al.* **Cloning of human ABCB11 gene in E. coli required the removal of an intragenic Pribnow-Schaller Box before it's Insertion into genomic safe harbor AAVS1 site using CRISPR-Cas9 [version 1; peer review: 2 approved]** F1000Research 2020, 9:1498 <https://doi.org/10.12688/f1000research.26659.1>

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Introduction

Progressive familial intrahepatic cholestasis type-2 (PFIC2), a severe liver disease which is familial, neonatal, progressive and often fatal which results from a mutation of ATP binding cassette subfamily B member 11 (*ABCB11*) gene which codes for an ABC transporter bile salt export pump (BSEP)^{1,2}. Mutations in *ABCB11* gene results in accumulation of cytotoxic taurocholate and other cholates conjugates leading to progressive hepatocyte destruction¹. Currently the definitive cure for PFIC2 is liver transplantation, which is limited by suitable donor organs. Gene therapy, allogenic hepatocyte transplantation³ and autologous transplantation of hepatocytes/liver organoids differentiated from ‘gene corrected’ induced pluripotent cells could be future options^{4,5}. Adeno-associated virus (AAV) is so ubiquitous in man and animals that about 30% of the world population are positive for this virus and to date no disease is proven to be associated with this virus⁶⁻⁹. In our study we inserted *ABCB11* gene at the AAVS1 site using CRISPR-Cas9 tool (Figure 1) in HEK293T cells and a fibroblast line.

Methods

Cloning of *ABCB11*

We PCR-amplified the 3966 bp *ABCB11* (from cDNA prepared from total RNA of human liver tissue) using multiple overlapping primers (Extended data, Supp. Table 1)¹⁰ which were assembled by overlap extension PCR¹¹. Phusion DNA polymerase (NEB, US Cat. #M0530L) was used as per the manufacturer's protocol. Annealing temperature for all PCRs unless otherwise stated was 60°C for 20s and an extension time 30s/kb at 72°C. Initial denaturation was carried out at 98°C for 30s and 5s in

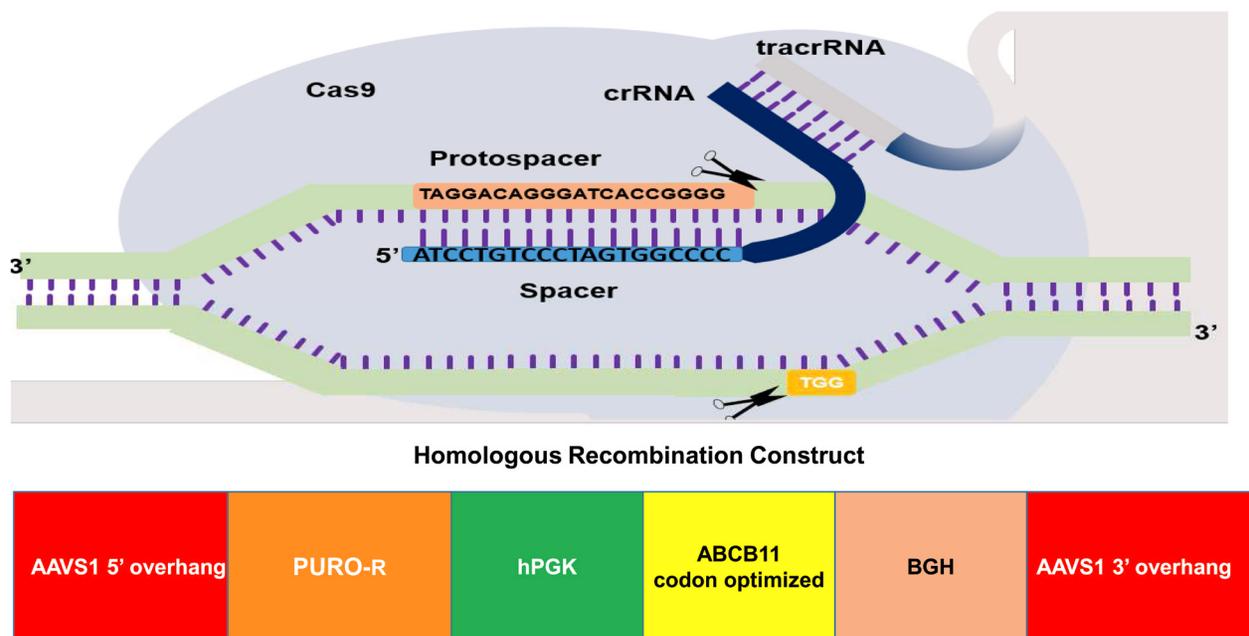
subsequent steps. In general, we used 24 cycles to amplify PCR products for cloning (and 32 cycles for other PCRs). We used 1.0 unit of the enzyme per 50 µl PCR reaction. The product was cloned in ‘donor’ vector having AAVS1 recombination overhangs on both ends and ampicillin resistance for selection (Extended data, Supp. Figure 1)¹⁰. The upstream (5′) overhang sequence (803 bp) in the vector was homologous to a sequence (NCBI Sequence ID: NC_000019.10, 55115768 to 55116570) inside the *PPP1R12C* gene (Figure 1), which would be in-frame with a 2A ribosome skip sequence and puromycin resistance gene if insertion of the construct happens by homologous recombination with the target site. This region was followed by a polyadenylation signal and the downstream (3′) overhang sequence which was the continuation of the 5′ overhang (837 bp). We inserted the *ABCB11* sequence driven by human phospho-glycerol kinase (hPGK) promoter between these overhang sequences. DH5α cells, JM 109 and One Shot™ Stbl3™ Chemically Competent *E. coli* cells (ThermoFisher Catalog#:C737303) were used for transformation and cloning.

Bioinformatics

Bacterial promoter prediction was done using **BROM**, a prediction tool for bacterial promoters¹². DNA or protein sequence comparisons were done using the appropriate tool from NCBI BLAST platform¹³. Primers were designed manually or using NCBI Primer Blast¹⁴.

gRNA

We designed two guide RNAs targeting the AAVS1 site (Figure 1) and cloned in two vectors expressing SPCas9 and



Information Classification: General

Figure 1. The Cas9-sgRNAs designed and the recombination cassette (donor vector). The gene of interest (*ABCB11*) was flanked by 5′ and 3′ overhangs which are homologous to the AAVS1 site in chromosome 19. *ABCB11* gene was driven by human promoter phospho-glycerol kinase.

SACas9 at BsaI sites (*Extended data*, Supp. Figure 2)¹⁰ following the standard gRNA design, cloning protocols and resources¹⁵. Off-target analysis was done using the tool Custom Alt-R@CRISPR-Cas9 guide RNA¹⁶.

Cell culture

HEK293T, HepG2 and FS1 (fibroblast) cells were grown in high glucose DMEM (Hi-Media Lab, Mumbai, Cat.# AL111-500ML) supplemented with 10% fetal bovine serum (CellClone, Genetix Biotech Asia, New Delhi, Cat.# CCS-500-SA-U), 1x penicillin (100U/ml) and streptomycin (100 µg/ml) (Hi-Media, Mumbai Cat. # A018-5X100ML). When 80% confluent, the cell lines were transfected with Cas9-sgRNA vectors (without the donor vector). At 48 h post-transfection, about 10000 of these cells were used for comet assay¹⁷ and genomic DNA (gDNA) isolated from the remaining cells was used for T7-endonuclease assay¹⁸ to evaluate the *in vitro* 'DNA cutting' activity of Cas9-sgRNA construct. Subsequently, we transfected these cells with the donor vector, Cas9-sgRNA vector and a control vector (pEGFPN1) in the ratio: 2:1:1 using PEI¹⁹ from Sigma Aldrich, Inc. (CAS #9002-98-6). After 48 h the cells were imaged and used for downstream applications. Half of the transfected dishes were serially passaged without puromycin selection for two weeks and DNA and protein were isolated. On the remaining dishes puromycin selection was started following the manufacturer's protocol²⁰ after 36 h of transfection and puromycin resistant colonies at 8 µg/mL were further cultured in puromycin containing media and gDNA was isolated.

Western blot

Whole-cell extracts (see *Cell culture*), scraped out and extracted using RIPA Lysis and Extraction Buffer, were run on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus following the standard protocols (Bio-Rad). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with TBST and incubated with rabbit antibodies against human ABCB11 (Affinity, Catalog #DF 9278) 1: 2000 dilution; human β-actin (Santa Cruz Cat.# SC4778), dilution 1:1000; 4°C overnight. The membrane was washed with TBST buffer and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit (Santa Cruz Cat# SC-2004)/anti-mouse antibodies (Cat.#SC-2005) for 2 h at room temperature. Blots were washed with TBST four times and developed with the ECL system (Bio-Rad, US Cat.#170-5060) according to the manufacturer's protocols. Raw, uncropped images from western blotting are available as Underlying data²¹.

T7 Endo Nuclease Assay

T7 endo I assay detects heteroduplex DNA that results from annealing DNA stands that have been modified after a sgRNA/Cas9 mediated cut to DNA strands without modifications. T7 Endonuclease-1 was purchased from NEB, US (Cat. #NEB #E3321) and was used to digest the PCR products amplified from gDNA extracted from Cas9-sgRNA transfected (test) and un-transfected

cells (control) using primers flanking the expected Cas9-sgRNA cut sites following the manufactures protocol¹⁸. PCR gel images are available as *Underlying data*²¹.

Comet assay

A total of 50–100 cells treated as described in *Cell culture* were embedded in 0.7% low-melting agarose and mounted on a pre-coated slide and was immersed in alkaline 0.1% SDS solution overnight, neutralized and electrophoresis was done in an alkaline buffer (pH 10) at 0.74 V/cm for 30 minutes¹⁷. Comet assay images are available as *Underlying data*²¹.

Sequencing

Sequencing of PCR products and plasmids were conducted by Invitrogen Bioservices India Pvt. Ltd., a part of Thermo Fisher Scientific, Whitefield, Bengaluru, PIN 560 066, India and Medauxin, Bengaluru, AMCO Colony, Koti Hosahalli, Bengaluru, PIN 560 092, India.

Results

Human ABCB11 gene/product is toxic to *E. coli* cells

Few ampicillin resistant DH5α *E. coli* colonies which we got after transformation were screened for the insert by colony PCR. One colony was positive for all the fragments of the ABCB11 gene. Sequencing revealed that mutations in ABCB11 (*Figure 2*). Repeated attempts failed and we considered the possibility of unstable DNA sequences. Therefore, we tried JM109 which gave one positive colony and plasmid was isolated. However, after we soon found the bacteria failing to grow or losing the plasmid on subsequent cultures. Therefore, we transformed One Shot™ Stbl3™ Chemically Competent *E. coli* cells, which are suitable for cloning unstable DNA segments. We got many positive colonies, however, upon overnight culture the bacteria formed a big pellet (partly lysed bacteria) which cannot be resuspended in phosphate buffered saline. Therefore, we concluded that the ABCB11 gene/gene product is toxic to bacterial cells. It is possible the ABCB11, being a membrane transporter, may be toxic to bacteria. Sequencing files are available as *Underlying data*²¹.

Identification and removal of a Pribnow-Schaller Box in human ABCB11 for cloning in *E. coli*

We conducted PAGE followed by Coomassie staining to see differential protein expression between ABCB11 donor vector transformed bacteria versus untransformed bacteria (*Figure 3a*; *Extended Data*)²¹. We found differential expression of a few proteins. We subsequently performed a western blot and interestingly antibody against human ABCB11 identified a specific protein over expressed in the transformed bacterial cells (*Figure 4b*). However, in our construct ABCB11 gene was under a eukaryotic promoter. Considering the possibility of some DNA elements which have similarity to bacterial promoters inside the ABCB11 sequence we performed a bioinformatic analysis using BPROM to predict hidden bacterial promoters (*Extended data*, Supp. Table 2)¹⁰. The promoter-site (Pribnow-Schaller box *tcataataat*) containing sequence (ggttttgatcagataaatcatataataat) which we identified

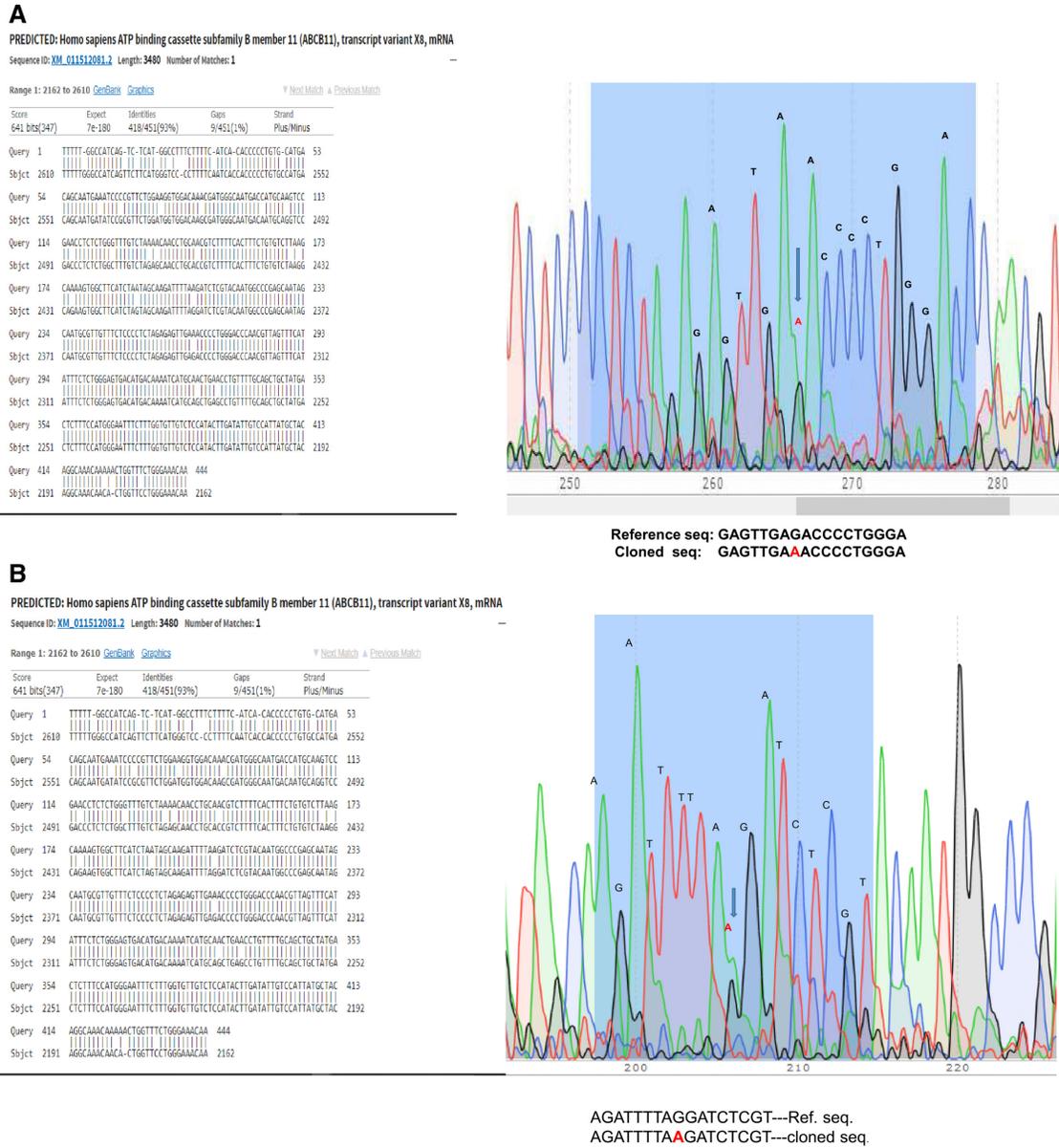


Figure 2. The ABCB11 gene cloned into E. coli showed mutations despite multiple cloning attempts. (a) G to A mutation is marked for example. (b) In another example, sequence of another clone, mutation was in a different site.

was modified to (ggTTCGAAtcagataatcaTACAACaat) by PCR using an oligonucleotide primer sequence incorporating the modified sequence and subsequent overlap extension PCR amplification of the entire gene fragment. With this modification, we were able to clone ABCB11 coding sequence which was not toxic to bacteria.

ABCB11 is similar to E. coli lipid A (endotoxin) transporter MsbA

A protein BLAST search identified MsbA (UniProtKB - P60752), a member of the ABC transporter superfamily. MsbA, a 64.46 kD protein, has an important role in E. coli Lipid A (endotoxin or LPS) transport (Figure 3c, d). This protein flips core

endotoxin from its site of synthesis on the inner leaflet of the inner membrane to the outer leaflet of the inner membrane. western blot showed identified a unique band in the donor vector transformed E. coli while the untransformed E. coli also showed a faint band but specific band at the same position (Figure 3b).

Verification of the gDNA cutting activity of Cas9-sgRNA construct

T7 Endonuclease Assay and Comet Assay were used to evaluate the gDNA cutting activity of Cas9-sgRNA. We observed digestion of heteroduplexes at the CRISPR-Cas9 cut sites which were sensitive to T7 endonuclease (Figure 4a). These heteroduplexes were observed on the agarose gel electrophoresis of

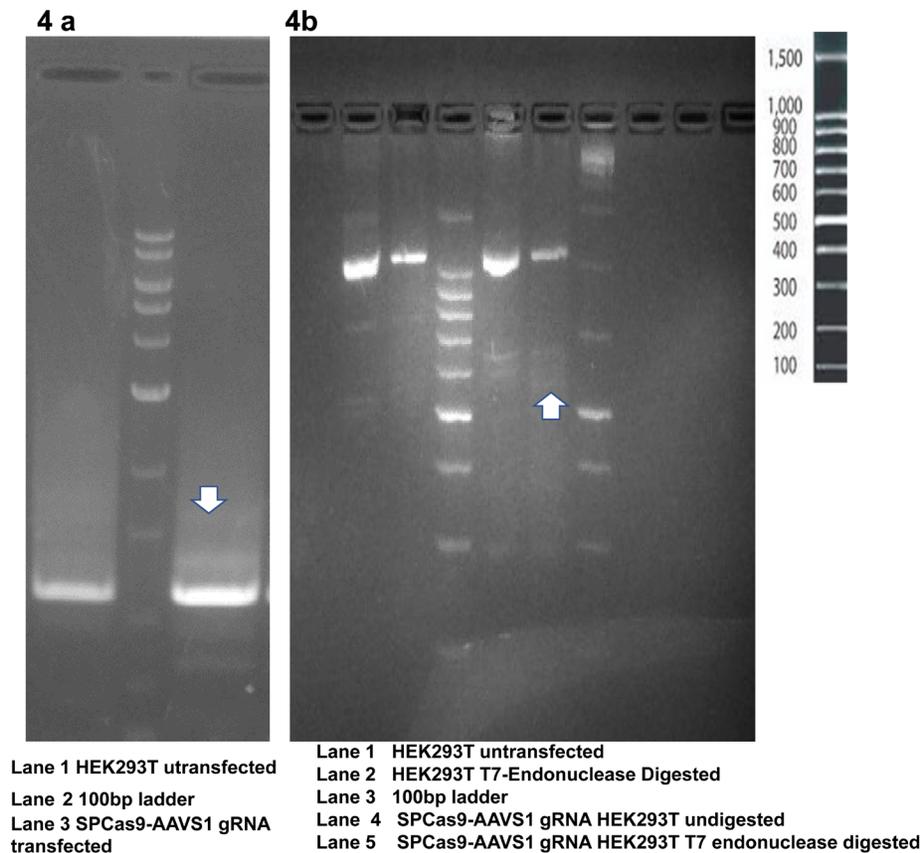


Figure 4. Agarose gel electrophoresis of PCR amplified products. **4a** The PCR product (even without T7 digestion) shows a distinct band pattern resulting from the formation of heteroduplex. **4b**. PCR amplified product after digestion with T7 endonuclease shows faint bands resulting from the digestion of heteroduplexes.

PCR products as well (Figure 4b). The Cas9-sgRNA damaged the genome of the transfected cells leading to the formation of comet shaped nuclear material upon electrophoresis (Figure 5).

Off-target analysis

Oligonucleotide PCR primers were designed for bioinformatically predicted off-targets (Extended data, Supp. Tables 3a, b)¹⁰. We amplified these segments using genomic DNA extracted from the treated cells (48 h post-transfection with SPCas9-sgRNA vector) as template. The PCR products were sequenced and analyzed for sequence disruption (Table 1).

Validation of ABCB11 expression in a fibroblast cell line

Western blotting done with total protein extract of fibroblasts 48 h post-transfection with the *ABCB11* donor vector showed the expression of ABCB11 protein (Figure 6a). A fibroblast line was used in these experiments because they don't express ABCB11, naturally while cell lines such as HEK293T and liver cell lines such as HepG2 do express ABCB11.

Western blotting was repeated with total protein extract from fibroblasts after 2 weeks (fourth passage) post-transfection with SPCas9-sgRNA vector together with the donor vector containing *ABCB11*. This blot also showed the expression of ABCB11

protein (Figure 6b) suggesting the integration of *ABCB11* into the host genome.

PCR amplification and sequencing to verify the integration of ABCB11 to AAVS1 site

We obtained only three puromycin resistant colonies upon transfecting about 20 million HEK cells with a transfection efficiency of 70 to 80% in four 6 cm dishes. The gDNA isolated from transfected cells (Cas9-sgRNA plasmid alone) after 72h, (Cas9-sgRNA plasmid plus donor vector) after 21 days of puromycin selection were used as PCR templates with a forward primer complementary to a region upstream of the 5' recombination overhang of the vector and a reverse primer complementary to a sequence in the puromycin resistance gene to amplify a segment spanning from a site in the host cell genomic DNA slightly upstream of the genomic integration site to a segment donated by the donor vector (puromycin resistance gene). This PCR product was used as a template for a nested PCR and product was confirmed by restriction enzyme digestion (BamHI) and sequencing (Primers: Extended data, Supp. Table 4¹⁰, Figure 7). We also PCR-amplified parts of ABCB11 using primers (Extended data, Supp. Table 1)¹⁰ which would give specific PCR products from the inserted cassette, to make sure the gene is not deleted from the cassette integrated to the host

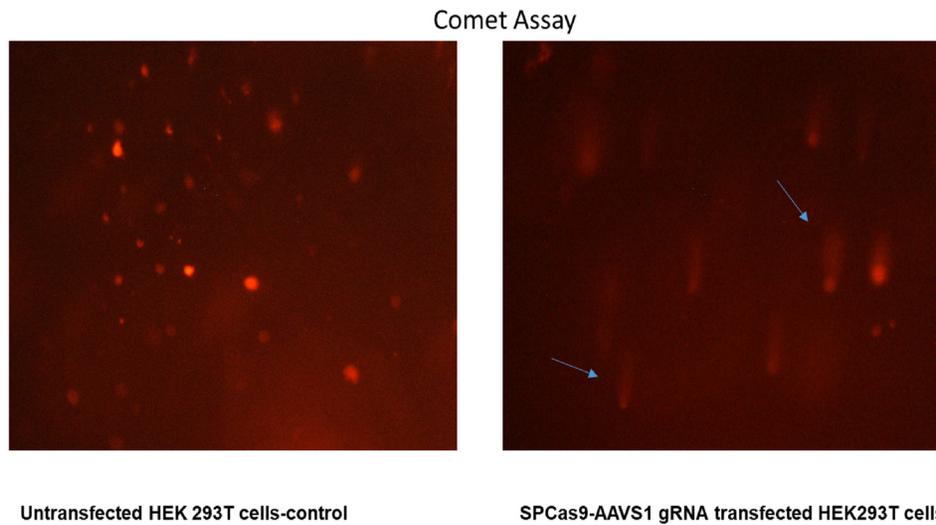


Figure 5. Cas9-sgRNA cause DNA damage, revealed by a Comet assay. The untreated cells have intact round/oval nuclei while the Cas9-sgRNA treated cells shows a comet shaped nucleus because of DNA damage.

Table 1. Selected off targets for crRNA GGGGCCACTAGGGACAGGAT (PAM TGG). Oligonucleotide primers designed to amplify these off-target sites to verify off target disruptions.

Off-Targets	Genes which may be affected	Primers	Size (bp)	Disruption
chr10: +119439168	G protein-coupled receptor kinase 5	F-AGCCTCCATCCAGATCCTGT R-TGGCCACAGTGTGTTTCCT	655	No
chr6: +36797686	copine-5 isoform e	F-TCCCAGTCTGCCCTCTCTTT R-CATGCCTCACTGCTCTACA	716	No
chr12- GRCh38.p13	MMP17 flanks 3'	F-GGGTCTCTGCTCTGAAACC R-TGGGAGATCTGGGAGAGGG	791	Yes
chr18: +8749293	microtubule cross-linking factor 1	F-GCCGTCAAATGGCACACAT R-TTGATCTGCCAAGCTCAG	630	No
chr20-GRCh38.p13	TMEPAI isoform d flanks 5'	F-ATCAGTCTGCTGCTACCCCT R-ATGAGGGGTCAGCCTATGGT	471	No
chr9-GRCh38.p13	Di-Ras2 flanks 3' PRO21346 flanks 5'	F-CCTCTGCCTTGCTGCTTTG R-TTGCTGCTGACACCTCCT	455	No

cell. PCR products showed the expected sizes confirming that the amplified products are from the cassette and not from the native *ABCB11* gene present in the cell line (Figure 8).

Discussion

To our knowledge, this is the first time the *ABCB11* gene was inserted into the AAVS1 safe-harbor using CRISPR-Cas9 technology in human cells. Liver directed gene therapy is another approach and was successful in rodents²². Adeno associated vectors do not integrate and therefore the effect of gene therapy many

not last in human beings, especially in infants, as the viral vector dilutes out as the cells proliferate in a growing liver²³. Another approach is transplantation of hepatocytes differentiated from gene corrected patient iPSC^{4,24,25}. AAVS1 site is considered as a 'safe haven' in human genome²⁶ where we chose to insert the gene mutated in PFIC. We could not find any other study which attempted to insert the *ABCB11* gene at the AAVS1 site. AAV is a common virus and it is considered non-pathogenic because the seroprevalence of wild-type AAV in humans ranges from 40% for AAV8 to 70% for AAV1 and AAV2, yet; we are not aware of

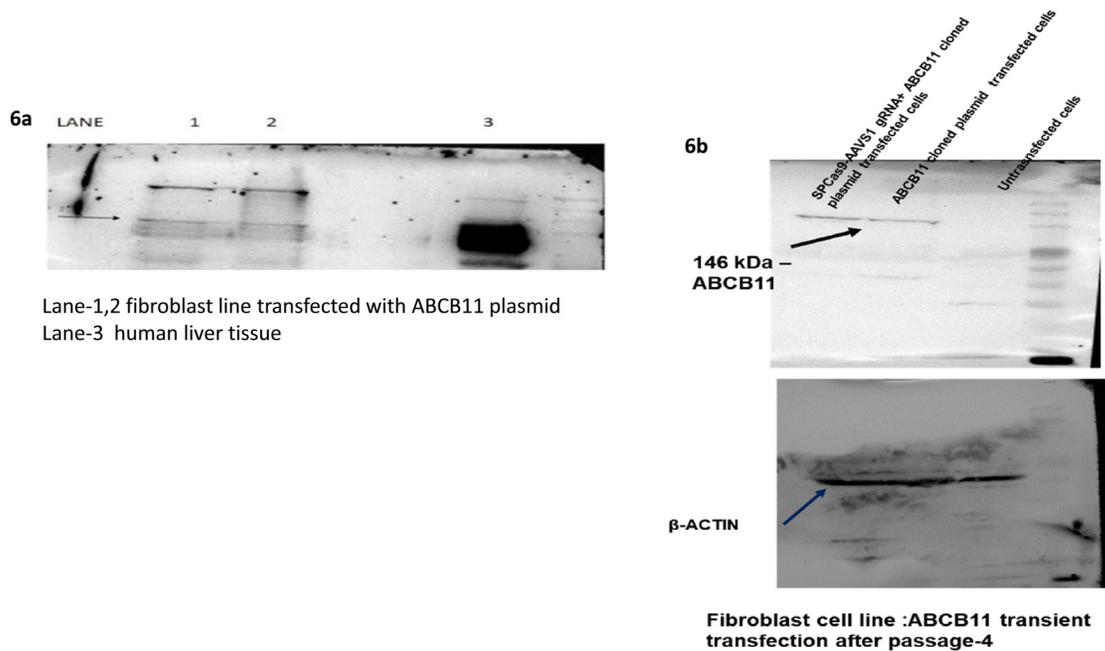


Figure 6. (a) Western Blot with anti-human ABCB11 antibody confirmed the expression after transient transfection with the donor vector having *ABCB11*. (b) Western Blot was done using total protein isolate from fibroblasts (which does not naturally express *ABCB11*) after four passages post-co-transfection of the donor vector with *ABCB11* gene and the CRISPR-Cas9-sgRNA vector.

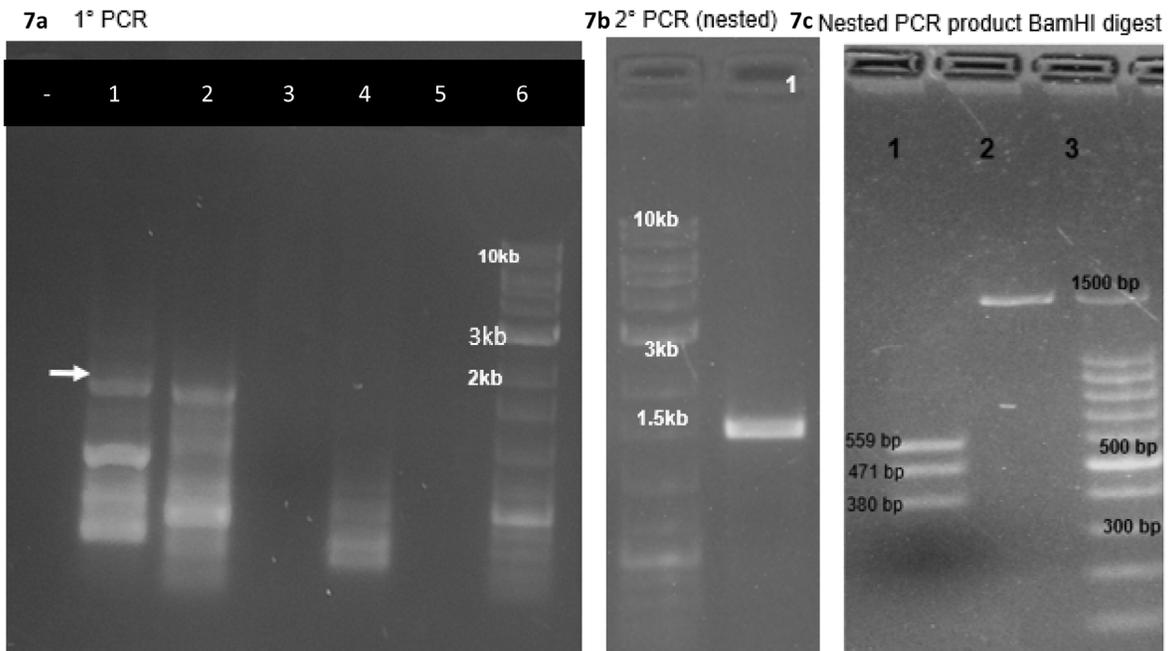


Figure 7. (a) The gDNA isolated from transfected cells (Cas9-sgRNA plasmid alone) after 72h, (Cas9-sgRNA plasmid plus donor vector) after 21 days of puromycin selection were used as PCR templates (lane 1, 2) with a forward primer complementary to a region upstream of the 5' recombination overhang of the vector and a reverse primer complementary to a sequence in the puromycin resistance gene to amplify a segment spanning from a site in the host cell genomic DNA slightly upstream of the genomic integration site to a segment donated by the donor vector (puromycin resistance gene). Note that genomic DNA from untreated HEK293T cells did not give any products in the expected range (lane 3, 4). (b) The PCR product mentioned in (a) was used as a template for a nested PCR. (c) The PCR product mentioned in (b) was confirmed by restriction enzyme digestion (BamH1) and sequencing (Primers: Extended data, Supp.Table 4)¹⁰.

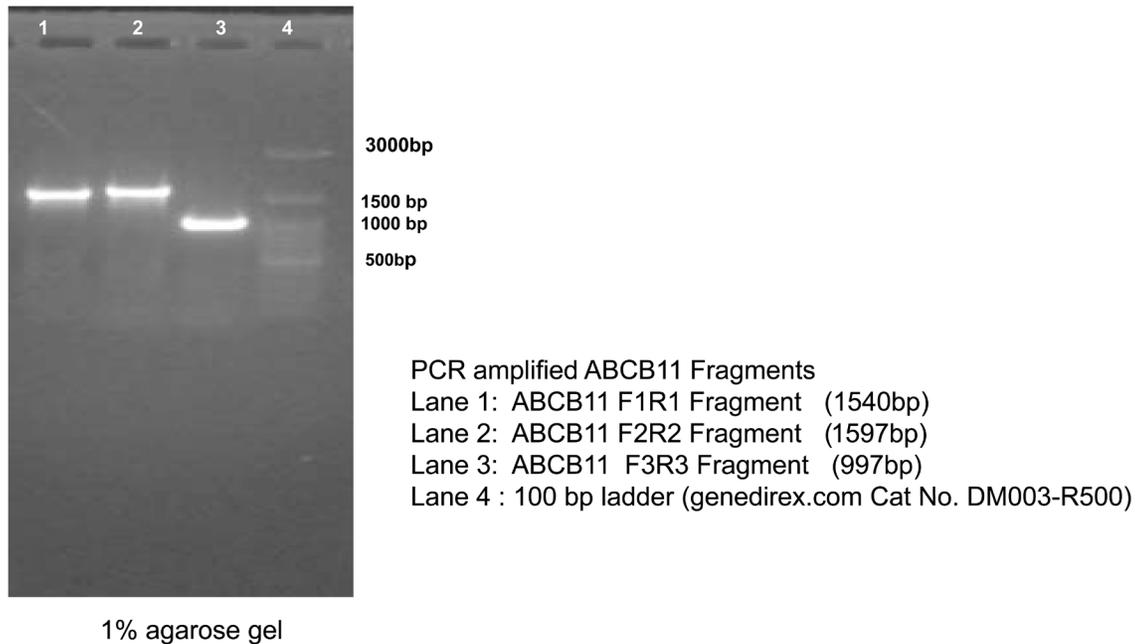


Figure 8. PCR products amplified from the genomic DNA. *ABCB11*-specific primers were used, which would give specific PCR products from the inserted cassette. This was done to make sure the *ABCB11* gene was not deleted from the cassette integrated to the host cell genome. The PCR products showed the expected sizes confirming that the amplified products originated from the integrated cassette and not from the native *ABCB11* gene present in the cell line.

any disease caused by AAV^{6,7,27}. AAV integration into AAVS1 site causes disruption of PPP1R12C (protein phosphatase 1 regulatory subunit 12C). However, this gene is not associated with any disease²⁷. A puromycin gene was placed in the donor cassette such that the puromycin gene will be transcribed only if homologous recombination happens. We obtained only a few puromycin resistant colonies suggesting that homologous recombination was a rare event ($\sim 10^{-7}$). This suggests that *in vivo* gene therapy using CRISPR-Cas9 technology making use of homology directed gene repair could be difficult. “Targeted Integration and high transgene expression in AAVS1 Transgenic mice after *in-vivo* hematopoietic stem cell transduction with HDAd5/35++ Vectors”²⁸ is reported; however, to achieve this they used an adenoviral gene delivery system with AAV5 ITRs and AAV35 helper. Integration of AAV/ Cas9 into Cas9 mediated cut sites is a potentially hazardous consequence of this approach^{29,30}.

We found that the human *ABCB11* donor vector transformed bacteria either died or the *ABCB11* gene sequence got mutated meaning either the DNA sequence or the ABCB11 protein has some untoward effects on bacteria. We performed a western blot and found that in ABCB11-transformed bacterial clones giving a band around 45 kD and HepG2 cells/liver tissue is giving a band at around 140 kD which corresponds to ABCB11. It was interesting to note that untransformed *E. coli* cells are also showing a band although very faint around 45 kD which suggested

the possibility of a bacterial protein which might have structural similarity to human ABCB11. We performed a bioinformatic search and identified MsbA an *E. coli* protein which functions as a lipid transporter (~64 kD). MsbA is involved in the transport of bacterial endotoxin-a function like the ABCB11 which transports the bile salts which are lipid derivatives^{31,32}. Another interesting observation was the identification of a bacterial promoter sequence (Pribnow-Schaller Box) in human *ABCB11* causing unexpected expression of ABCB11 protein and bacterial toxicity (Figure 9). This was an important lesson for us because we spent a lot of time trying to clone ABCB11. It is therefore important to search for and eliminate if any bacterial promoter sequences or similar elements are identified, for successful cloning of eukaryotic/toxic genes in *E. coli*. We do not know how exactly ABCB11 caused bacterial toxicity. Possibly, expression of ABCB11 in *E. coli* might be destabilizing the *E. coli* membrane, since ABCB11, being a lipid transporter, is a membrane-spanning protein.

Alternatively, the human protein, which is similar to the *E. coli* protein might have caused a competition between the bacterial transporter MsbA and human protein for membrane incorporation resulting in the accumulation of endotoxin within *E. coli* cells because unlike the bacterial transporter the membrane incorporated ABCB11 might not be able to transport endotoxin out. This raises the possibility that endotoxin is toxic to

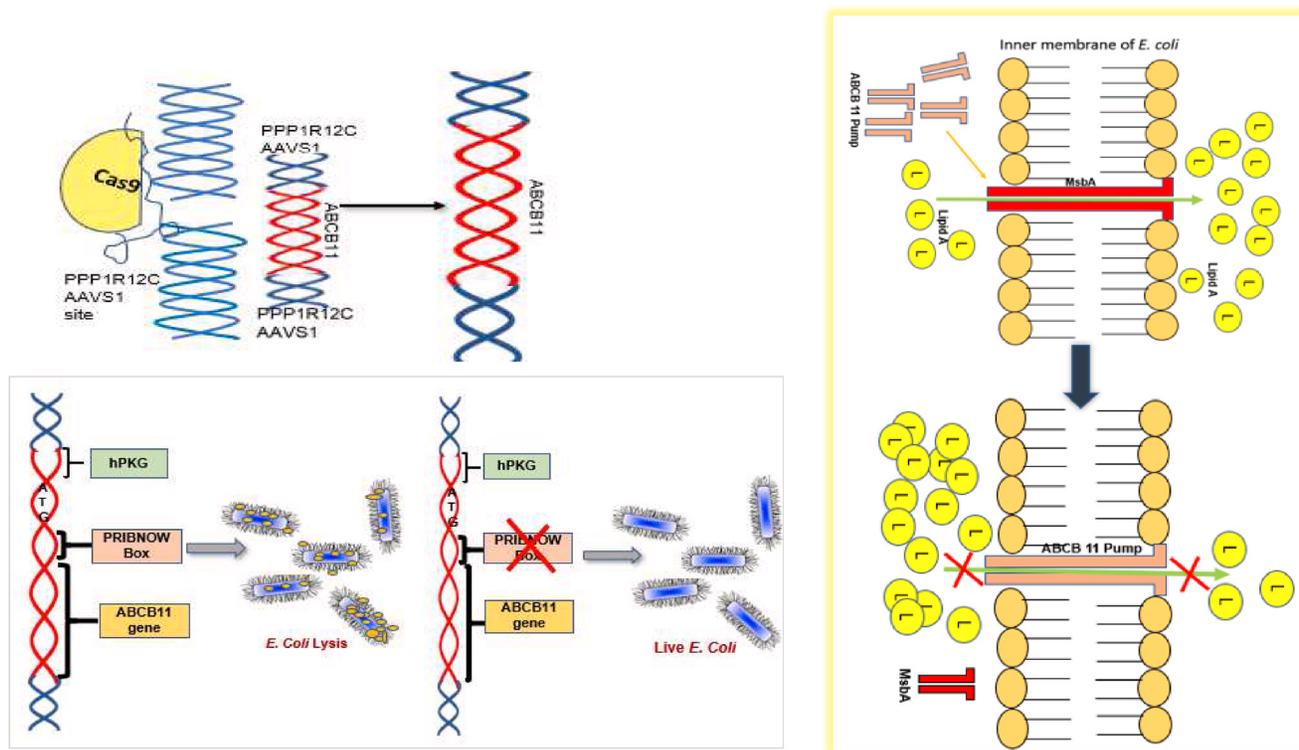


Figure 9. The *ABCB11* gene (which codes the transporter of human bile salts) is targeted to AAVS1 site using a construct which has 5' and 3' overhangs which are homologous to the AAVS1 site. A Pribnow box was detected inside *ABCB11*, which allowed the gene to transcribe in *E. coli*, causing bacterial lysis, probably through competitive replacement of a homologous transporter protein in *E. coli* (*E. coli* Endotoxin (Lipid A) Transporter) MsbA, resulting in Lipid A (L) accumulation inside the bacteria.

E. coli itself if it is not exported and MsbA can therefore be considered as a drug target. This point required further validation (Figure 9). The advantage of such an antibiotic is that it will be selective to endotoxin producing microbes. More research is required in this direction. It may be noted that endotoxin producing microbes play an important role in sepsis³³ and diseases such as non-cirrhotic portal hypertension^{34–36}.

To conclude, we successfully inserted *ABCB11* at the AAVS1 site using CRISPR-Cas9, however, the frequency of homologous recombination was very low as evident from the number of puromycin resistant colonies. With this low efficiency, with the current technology it is unlikely that this approach would be successful in *in-vivo* gene editing. It is worth, exploring MsBA as a novel antibiotic target for LPS producing bacteria, although our data in this direction is primitive and requires further validation.

Data availability

Underlying data

Harvard Dataverse: Sequencing Data, supplementary data to Cloning of Human *ABCB11* Gene in *E. coli* required the removal of an Intragenic Pribnow-Schaller Box before it's Insertion into Genomic Safe Harbor AAVS1 Site using CRISPR Cas9. <https://doi.org/10.7910/DV/N/32TXCD²¹>.

This project contains the following underlying data:

- 2020_06_20_211607 actin.jpg. (Uncropped western blot image.)
- 4a .T7 endonuclease digestion.jpg. (PCR gel image.)
- 4b. PCR without T7 endonuclease.jpg (PCR gel image.)
- 4c... Cas9-sgRNA treated cells comet assay.jpg. (Image taken from Comet assay, treated cells.)
- 4c..... untreated cells comet-1.jpg. (Image taken from Comet assay, untreated cells.)
- *ABCB11*_fibroblasts.jpg. (Uncropped western blot image.)
- *ABCB11*_fragments.tif. (PCR gel image.)
- *ABCB11*_WB_P4_2020_06_19_183004-1.tif. (Uncropped western blot image.)
- DH5a WB-*ABCB11*-long_Exposure.jpg. (Uncropped western blot image.)
- DH5a WB-*ABCB11*.jpg. (Uncropped western blot image.)

- E.Coli_PAGE_Coumasse.jpg. (Uncropped PAGE gel.)
- JM109_ABCB11 WB.tif. (Uncropped western blot image.)
- nested PCR product BamH1 digest.jpg. (PCR gel image.)
- Nested PCR secondary.jpg. (PCR gel image.)
- Nested_Primary pcr.jpg. (PCR gel image.)
- Repeat_WB_2020_07_11_181831.jpg. (Uncropped western blot image.)
- Sequencing data.7z. (Sequencing data produced in the present study.)
- T7endo 30420202.jpg. (PCR gel image.)

Extended data

Harvard Dataverse: Supplementary Tables to Cloning of Human ABCB11 Gene in E. coli required the removal of an Intragenic Pribnow-Schaller Box before it's Insertion into Genomic Safe Harbor AAVS1 Site using CRISPR Cas9. <https://doi.org/10.7910/DVN/NTUOXM¹⁰>.

This project contains the following extended data:

- Supp-Tables-revised-HepInt.docx. (Supp. Tables 1–4.)
- SuppFigures.pptx. (Supp. Figure 1a, b.)

Acknowledgements

The corresponding author thanks Mr. Rahul Saha for his assistance in western blot.

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<https://doi.org/10.5256/f1000research.29430.r81735>

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The manuscript is well-designed with a valuable contribution to the successful cloning of ABCB11, a key defective gene in PFIC2.

I only have a few minor comments:

- Is that common to see mutations in ABCB11 gene while expressing it in the prokaryotic or eukaryotic expression system?
- Do authors know the functional consequences if this gene is not mutated in the expression system?
- Title of manuscript is too long.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.**Reviewer Expertise:** Molecular Cell Biology**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 06 April 2021

<https://doi.org/10.5256/f1000research.29430.r81026>

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**Mitradas Panicker**

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This is an interesting and useful observation regarding the cloning of the human ABCB11 gene in *E.coli* prior to its original goal i.e. inserting ABCB11 into the AASV1 safe harbor.

The aim was to insert the ABCB11 gene into human cells to establish the initial steps for gene therapy in liver. The authors found that they could not clone their construct of the gene in *E. coli*. Careful observation and analysis led them to a Pribnow-Schaller Box within the sequence. They proved their hypothesis by modifying the Box and successfully cloning the gene in *E. Coli*. The work is detailed and carefully done and also suggests further areas such as the potential use of MsbA as a drug target.

Comments:

The title could be corrected to reflect the result 'proper' since the removal of the Pribnow-Schaller box is not a necessary requirement for insertion into the AASV1 locus. It became a necessity due the cloning method that had to be adopted.

The manuscript requires some additional editing for clarity.

Figure 1 would benefit by indicating the site of the 2A insertion, the hPGK sequence should be marked as a promoter.

It is not clear what is the ABCB11 codon optimization for and also what it was optimized for as noted in Fig.1.

It would be more accessible if the Pribnow-Schaller Box and the sequences changed are depicted as a figure than as a supplementary table and a few lines of text. If text is preferred then the

sequence numbers of the nucleotides involved and the reference sequence should be provided in the text.

The observation that homologous insertion was obtained at very low frequency does not necessarily preclude the strategy from being used 'in vivo' since the frequency would be highly dependent on the strategy and the sequences chosen. For e.g. a homology-independent strategy with same construct might prove fruitful. Perhaps the discussion could reflect this.

The supplementary Figure of the Donor Vector does not show the ABCB11 sequence. This should also be addressed.

The observation by Chang and Gray should be more clearly emphasized in the Discussion. MsbA as a drug target should also refer Zhang *et al.* (2018)¹.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Neurobiology, Stem Cells, Neuroscience, Cell Biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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