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Designing and Cloning Guide RNA Plasmids for Targeted Editing of Mammalian RNAs by Using CRISPR-Cas13b

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ABSTRACT

Background/Purpose: CRISPR/Cas13 expands the CRISPR/Cas9-mediated DNA editing approaches to the RNA editing. In this system, a guide RNA (gRNA) targets a specific region in the RNA of interest and recruits Cas13. gRNA can be designed with little restriction to cover almost the whole transcriptome and various engineered Cas13 enzymes with unique added features can be utilized at the region of interest.

Methods: Plasmids were obtained from Addgene plasmid repository and their integrities were first confirmed by restriction enzyme digestions. An oligo that is complementary to the region surrounding the start codon of *PATZ1* mRNA was designed and cloned into a gRNA plasmid by using a golden gate reaction. The cloned plasmid was confirmed by Sanger-sequencing.

Results: A 45-nucleotide long sequence that is complementary to *PATZ1* mRNA around the AUG start codon with a mismatched cytosine for the corresponding adenine at the 30th nucleotide from the 3' end of the sequence was designed and cloned into gRNA plasmid.

Conclusion: We designed and cloned a gRNA plasmid that targets the start codon of human *PATZ1* mRNA. When this plasmid is co-transfected into cells with a catalytically inactivated Cas13 fused to an adenosine deaminase encoding plasmid, the adenine nucleotide in the canonical start codon of *PATZ1* is expected to be edited to inosine. This change might be functionally important to study the decrease in protein translation or the truncation of N-termini in future studies.

Keywords: CRISPR, Cas13, ADAR, PATZ1, RNA editing

ÖZET

Giriş/Amaç: CRISPR/Cas13, CRISPR/Cas9 aracılı DNA düzenleme yaklaşımlarını RNA düzenlemede kullanıma izin verir. Bu sistemde, bir kılavuz RNA (gRNA) hedef RNA'daki belirli bir bölgeyi bulur ve Cas13'ü beraberinde getirir. gRNA, neredeyse tüm transkriptomu kapsayacak şekilde çok az kısıtlama ile tasarlanabilir ve benzersiz ek özelliklere sahip tasarlanmış çeşitli Cas13 enzimleri hedef bölgede kullanılabilir.

Yöntemler: Plazmidler Addgene plazmid deposundan alındıktan sonra ilk olarak bütünlükleri restriksiyon enzim kesimiyle doğrulandı. *PATZ1* mRNA'sının başlangıç kodonunu çevreleyen bölgeye tamamlayıcı olan bir oligo tasarlandı ve golden gate reaksiyonu kullanılarak bir gRNA plazmidine klonlandı. Klonlanan plazmid Sanger dizilimiyle doğrulandı.

Bulgular: *PATZ1* mRNA'sının AUG başlangıç kodonu çevresine tamamlayıcı olan 45 nükleotid uzunluğunda bir dizi, dizinin 3' ucundan itibaren 30. nükleotide karşılık gelen adenin için uyumsuz bir sitozin ile tasarlanmış ve gRNA plazmidine klonlanmıştır.

Sonuç: İnsan *PATZ1* mRNA'sının başlangıç kodonunu hedefleyen bir gRNA plazmidi tasarladık ve klonladık. Bu plazmid, adenozin deaminaz kodlayan bir plazmide kaynaşmış katalitik olarak inaktive edilmiş bir Cas13 ile birlikte hücrelere aktarıldığında, *PATZ1*'in kanonik başlangıç kodonundaki adenin nükleotidinin inozine düzenlenmesi beklenmektedir. Bu değişiklik, gelecekteki çalışmalarda protein translasyonundaki azalmayı veya N-ucu kırpık protein incelemek için işlevsel olarak önemli olabilir.

Anahtar Kelimeler: CRISPR, Cas13, ADAR, PATZ1, RNA Düzenleme

Engineered CRISPR/Cas systems are now widely applied for genome editing. Among these, the most famous is CRISPR/Cas9, in which a bacterial complementary CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) are combined into one intact RNA called as single guide RNA (gRNA) (1). gRNA binds Cas9, which is a DNA endonuclease, and guides it to the target DNA locus. In this system, gRNAs may require a predefined short stretch of flanking DNA sequence, which is called as protospacer adjacent motif (PAM). While gRNA binds to target DNA and forms a short hairpin, Cas9 is guided to this site to cleave the DNA. In eukaryotic cells, cleaved DNA initiates DNA damage response and cells edit the target DNA while repairing it (2).

Various other CRISPR/Cas systems were later discovered that utilize different Cas proteins or gRNA structures. CRISPR/Cas13 system has expanded the genome editing to RNA editing. This system takes advantage of the type VI CRISPR-associated RNA-guided ribonuclease Cas13 (3). Cas13 enzymes consist of four protein families that are smaller in size compared to Cas9: Cas13a, Cas13b, Cas13c and Cas13d (4). These enzymes cleave the target RNAs with their two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) endoRNase domains. CRISPR/Cas13 also requires a gRNA to bind to target sequence and this gRNA forms a hairpin structure near a protospacer flanking site (PFS) to guide Cas13. Subsequently, Cas13 cleaves the target RNA with the activated HEPN domains. This catalytic activity of CRISPR/Cas13 can be harnessed to knockdown RNA expression in eukaryotic cells in a way that is similar to RNA interference (5).

CRISPR/Cas systems are very versatile tools to be utilized for various molecular biology approaches. One of the engineered versions of CRISPR/Cas13 has a catalytically inactive or so-called "death" Cas13 ortholog from *Prevotella sp.* (dPspCas13b) that is still capable of being guided by gRNAs. Cox, et al., further engineered dPspCas13b by fusing a modified human adenosine deaminase acting on RNA 2 enzyme (ADAR2) to it (3). ADARs are enzymes that edit double stranded RNAs at adenine:cytosine (A:C) mismatches by converting A to inosine (I) to form I:C Watson-Crick pairs. Inosine is a nucleobase functionally equal to guanine (G) during RNA splicing and protein translation (6). The chimeric dPspCas13b - ADAR2DD [E488Q/T375G] protein is highly capable of editing target RNAs with very low number of off-targets and does not require any strict sequence constraints such as a PFS. With this type of precise RNA editing, disease causing mutations can be corrected, single nucleotide polymorphisms can be modelled, or protein translation start codons can be altered among many other applications.

In the current study, we designed and cloned a gRNA that would target the start codon of *PATZ1* mRNAs. Because PATZ1 is an important DNA damage responsive transcription factor and is an important inhibitor of the p53 tumor suppressor protein functions, studying the functions of PATZ1 is crucial for understanding tumorigenesis (7). The cloned plasmid may be used to destroy the canonical start codon of *PATZ1* mRNA.

Materials and Methods

Plasmids

pC0052 (REPAIR non-targeting guide clone into pC0043) (Addgene plasmid #103868), pC0043 (PspCas13b crRNA backbone) (Addgene plasmid #103854), and pC0054 (CMV- dPspCas13b- longlinker- ADAR2DD [E488Q/T375G]) (Addgene plasmid #103870) plasmids were gifts from Feng Zhang (3). MiniPrep Plasmid DNA isolation was performed using the alkaline lysis protocol (8).

Restriction Enzyme Digestion

The integrity of the plasmids was confirmed based on their expected fragment size patterns on an agarose gel after their digestion with different restriction enzymes. 500 ng of a plasmid was used with each restriction enzyme (New England Biolabs Inc.) and the reactions were performed according to the manufacturer protocols. The resulting DNA fragments were resolved on a 0.7% agarose gel for 60 min at 100V. Ethidium Bromide (EtBr) -stained DNA bands were captured using a ChemiDoc Imaging System (Bio-Rad).

Annealing of gRNA Insert

Two complementary DNA oligonucleotides were designed to express the gRNA against the target RNA, and flanked with additional nucleotides to create sticky ends: PspCas13bPATZ1top (5'- cac cCG TTC ACC CGC TCC A***c***G GCC GCC GCC CCC TCC CAC TAG CCC GGC C -3') and PspCas13bPATZ1bot (5'- caa cGG CCG GGC TAG TGG GAG GGG GCG GCG GCC ***g***TG GAG CGG GTG AAC G -3'). The oligos were annealed at 10µM final concentration for each in 10 µL final reaction volume with 1X T4 ligase buffer (NEB, #B0202S) and 1 unit/µl T4 Polynucleotide Kinase (NEB, #M0201S) by the following parameters (37°C 30min, 95°C 5min, ramp-down to 25°C (5°C / 5min)). The success of the annealing reaction was confirmed by resolving the single stranded and double stranded oligos on an agarose gel with EtBr.

Cloning of gRNA Insert into the Plasmid

The double stranded DNA oligo coming from the annealing reaction was diluted 1:200 with ddH_2O and 6µl of this dilution was mixed with 100 ng of pC0043, 2µl of 10X T4 ligase buffer, 10 units of BbsI restriction enzyme (NEB, #R0539S), 2µl of BSA (1µg/µl), 200 units of T4 DNA ligase (NEB, #M0202S) and ddH_2O up to 20µl total volume. Linearization of the plasmid followed by the ligation of the insert was performed by a golden gate reaction by the following parameters: (37°C 5min, 21°C 5min)x12. Unligated free oligos in the reaction mixture was later removed by treating the sample with exonuclease V. For this, the 20µl of ligation reaction yield was supplemented with 3µl NEBuffer 4 (NEB, #B7004S), 3µl of 10mM ATP (NEB, #P0756S), 10 units of T5 Exonuclease (NEB, #M0363S) and ddH2O up to 30µl total volume. The treatment was performed at 37°C for 30 min and stopped by supplementing 3.5µl of 100mM EDTA and 1.5µl ddH_2O followed by an incubation at 70°C for another 30 min.

Colony PCR

10µl of the golden gate reaction product was used to transform DH5α Chemically Competent E. coli. Eight colonies were picked from the agar plate and bacteria cultures were propagated from them for miniprep DNA using alkaline lysis protocol (8). For colony PCR, 1µl of the miniprep DNA was mixed with 14.5µl of ddH_2O, 2µl of 2.5µM U6 forward primer (5'- GAG GGC CTA TTT CCC ATG ATT CC -3'), 2µl of 2.5µM PspCas13bPATZ1bot primer, 5µl of 5X MyTaq buffer, and 0.5µL of 5u/µl MyTaq polymerase (Bioline, #BIO-21105). PCR was performed in a thermocycler machine with the following parameters: 95°C 1 min (95°C 15 sec, 62°C 15 sec, 72°C 18sec)x35. The PCR products were visualized by resolving the DNA samples on an agarose gel with EtBr. Selected plasmid clones were sent out for Sanger sequencing using U6 forward primer. Sequencing results were visualized and analyzed by using QIAGEN CLC Main Workbench bioinformatics tool.

Results

Confirmation of Plasmid Integrities

Three important plasmids are used for the dPspCas13b-ADAR2-mediated CRISPR/Cas13b RNA editing system. pC0043 is the backbone required for expressing the desired gRNA cloned into it. pC0054 is a eukaryotic protein expression plasmid that encodes dPspCas13b-ADAR2DD fusion protein in mammalian cells. pC0052 is actually identical to pC0043 except for an already cloned non targeting guide RNA sequence that has no possible target site on mammalian RNAs and is used as negative control. After the plasmids were isolated by miniprep from the transformed bacterial stab cultures purchased from Addgene plasmid repository**,** the plasmids were first run on an agarose gel to visualize their integrity (Fig. 1a). The most intense bands observed on the image pointed out the expected size of the corresponding plasmid and confirmed that plasmids were intact. The integrity of the plasmids was further assessed by restriction enzyme digestion. For this, the plasmids were digested using appropriate restriction enzymes that would produce distinctive DNA fragments (Fig. 1b). Accordingly, resolving the digestion reaction on an agarose gel revealed the expected DNA bands at the expected sizes and confirmed the plasmid integrities.

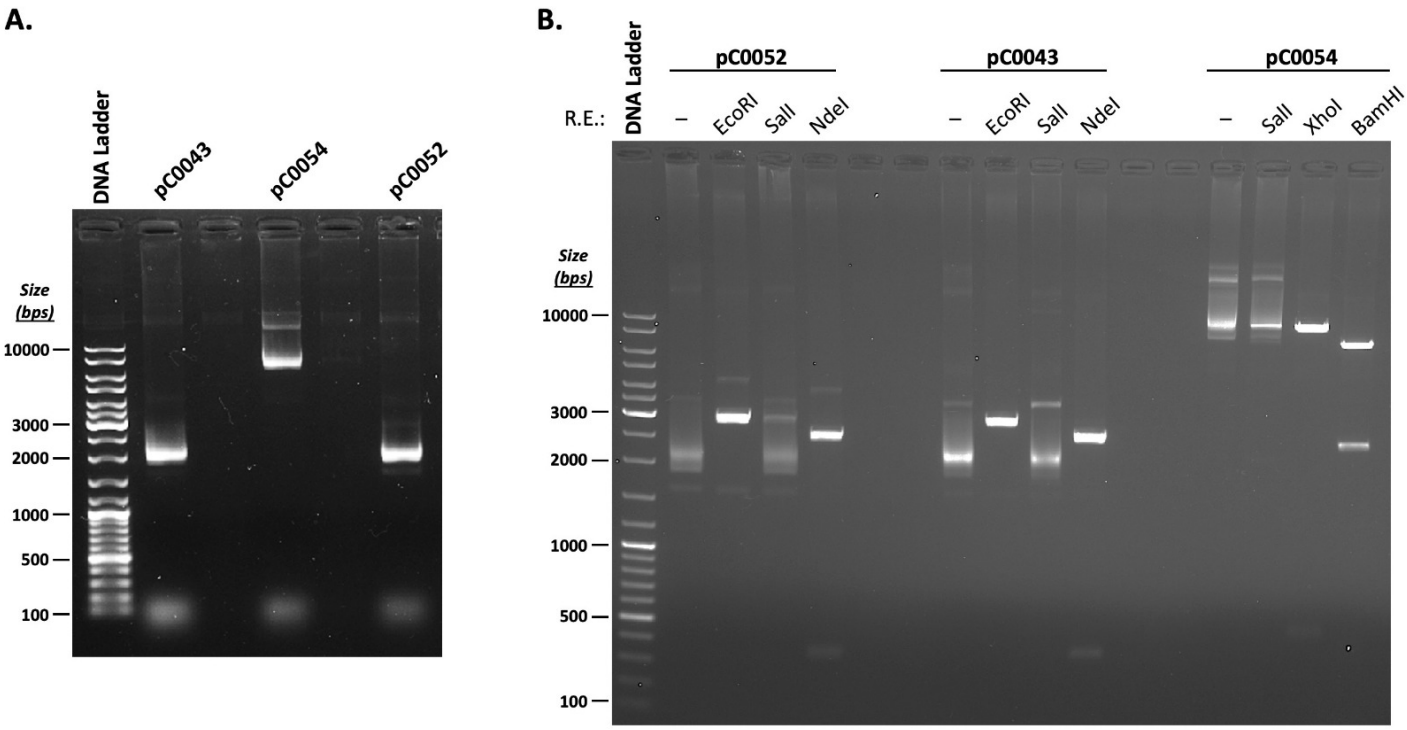

Figure 1: The plasmids required for CRISPR-Cas13b RNA editing system were intact. **A.** Miniprep DNAs were run on a 0.7% agarose gel for 60 min at 100V. EtBr-stained DNA bands were captured using a ChemiDoc Imaging System (Bio-Rad). Expected bands (bp: base pairs) are 2962bp for pC0043, 9864 bp for pC0054 and 2970 bp for pC0052. **B.** Plasmids were digested by restriction enzymes (R.E.) and DNA fragments were analyzed by agarose gel electrophoresis. For PC0052: EcoRI (2962bp), SalI (2962bp) and NdeI (372bp and 2602bp). For pC0043: EcoRI (2962bp), SalI (2962bp) and NdeI (364bp and 2602bp). For pC0054: SalI (2192bp and 7680bp), XhoI (489bp and 9383bp) and BamHI (2475bp and 7397bp). Note that the digestion reactions with SalI were apparently not to the completion. Also, DNA fragments shorter than 500bp were not easily visible under the current exposure settings.

Designing and Cloning Guide RNA Sequence

In order to edit the AUG start codon of *PATZ1* mRNA via deamination of adenosine to inosine, a gRNA that is complementary to the region encompassing the target adenine was designed (Fig. 2a). For this a 45-nucleotide long sequence was selected in such a way that the 30th nucleotide from the 3′ end of the gRNA sequence would correspond to the adenine of the AUG codon and this specific nucleotide was replaced with a cytosine in the oligo. The DNA oligonucleotide that is expected to encode this guide RNA from the cloned plasmid was created by adding additional overhangs for cloning purposes. For the cloning into pC0043 plasmid, firstly, two complementary DNA oligos were annealed that was confirmed by running the reaction yield on an agarose gel in the presence of ethidium bromide, which would bind DNA by exceedingly intercalating between base pairs (Fig. 2b). Subsequently, the double stranded DNA insert was cloned into pC0043 plasmid via golden gate reaction by digesting the plasmid with BbsI restriction enzyme and ligating the insert with T4 DNA ligase. The final plasmid construct was named as pC0043-PATZ1-gRNA. The sequence was annotated with all the important features and schematically represented in figure 2c.

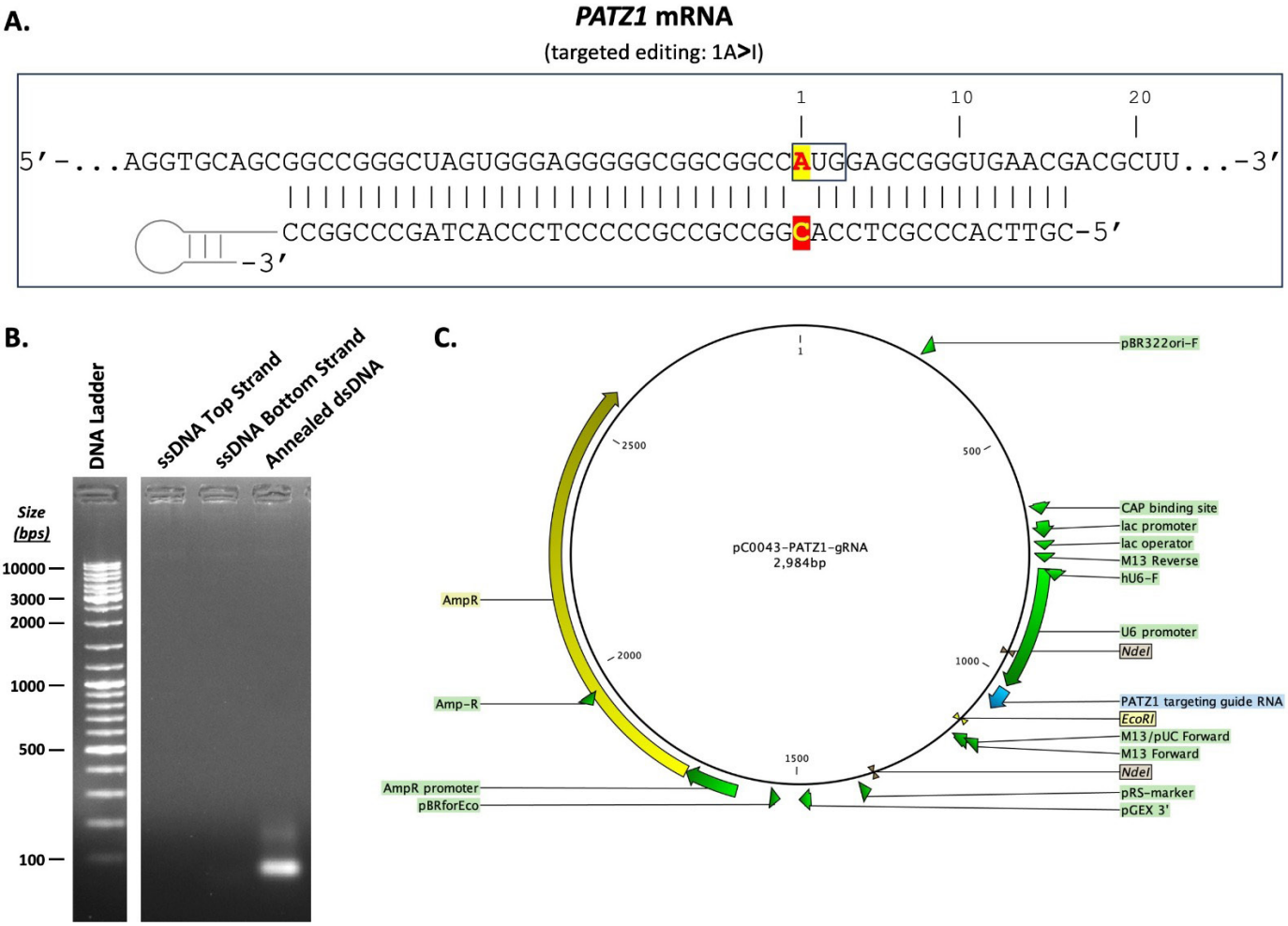

Figure 2: *PATZ1* mRNA targeting gRNA was designed and cloned into pC0043 plasmid. **A.** The mRNA sequence of *PATZ1* was acquired from Ensembl database (ENST00000266269.10). The gRNA sequence (below) complementary to the *PATZ1* mRNA (above) was shown. The adenine of the start codon (highlighted with a box) was numbered 1. **B.** The annealing success of the single stranded complementary DNA oligos encoding the gRNA was revealed by ethidium bromide staining of the agarose gel. **C.** The schematic representation of the final cloned plasmid, pC0043-PATZ1-gRNA, was generated by QIAGEN CLC Main Workbench software. Annotations on the map represent primer binding sites, antibiotic resistance gene, restriction enzyme cut sites and gRNA insert.

Confirmation of the Cloned Plasmid

The golden gate reaction was transformed into bacteria to select for individual clones. For this, first a colony PCR was performed to quickly screen for the presence of the insert using a PCR primer pair that includes an oligo that targets the U6 promoter as the forward primer and the specific bottom oligo used for the gRNA insert as the reverse primer (Fig. 3a). The colony PCR showed that all selected colonies from the agar plate contained the gRNA insert. Plasmids were extracted from the expanded bacteria cultures and submitted for Sanger sequencing using U6 forward primer. This gave greater insight into the confirmation of the exact insert sequence in the plasmids. 5 plasmid samples were sequenced in total but only the sequence of colony #2 is included here as a representative result (Fig. 3b). According to the sequence, the corresponding insert was missing one nucleotide at the downstream end that is next to the hairpin structure of the encoded RNA.

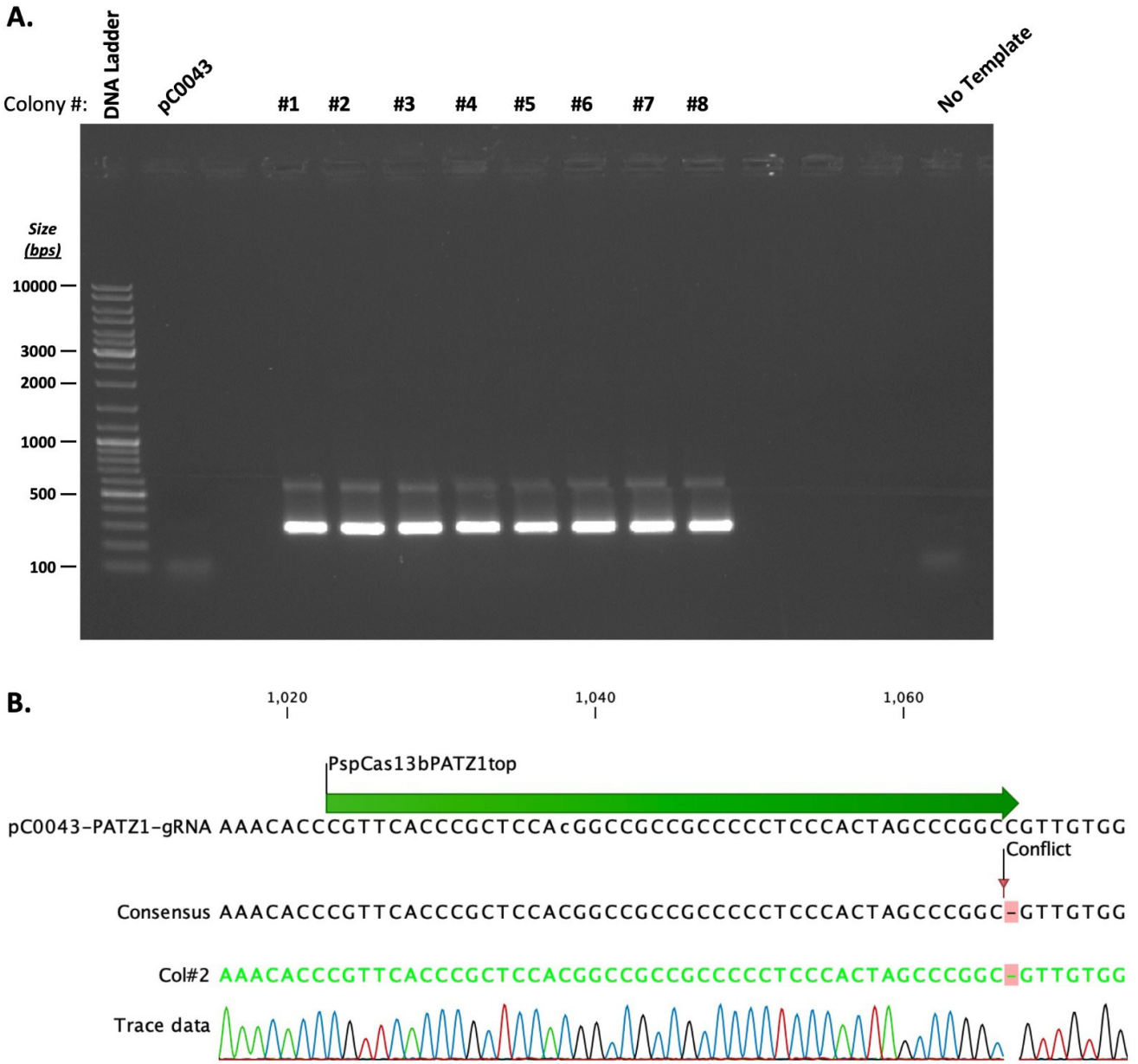

Figure 3: The final plasmid construct sequence. **A.** The transformed bacteria colonies were initially screened by using colony PCR with U6 forward and PspCas13bPATZ1bot reverse primers. A 298bp PCR product was expected from the successful transformants. **B.** The plasmids were extracted from the corresponding colonies using alkaline lysis miniprep method and submitted for sanger sequencing with U6 Forward primer. The colony shown here was missing the last nucleotide, which was indicated as a conflict on the alignment. Sequencing peaks were visualized by using QIAGEN CLC Main Workbench.

Discussion

There are catalytically impaired Cas9 proteins fused to adenine or cytidine base editors that can alter a specific start codon on DNA level (9). Alternatively, CRISPR/Cas13 system is another technology for altering gene expression by targeting, skipping or editing RNAs. This technology is advantageous over Cas9 as it does not damage DNA (10). PspCas13b, which was also used in the current study, does not require a predefined PFS sequence on the target RNAs, potentially making it to be used across the whole mammalian transcriptome (3). CRISPR/Cas13 was proven to eliminate or correct pathogenic mRNAs. Powell, et al., (11) utilized RfxCas13d, a Cas13 ortholog, to knock-down

mutant *superoxide dismutase 1 (SOD1)* mRNA, which otherwise cause amyotrophic lateral sclerosis, in mouse spinal cord and brain via adeno-associated virus delivery. They also targeted *huntingtin (HTT)* mRNA, which causes Huntington's disease, with a similar approach.

Adenosine deaminases acting on RNA (ADAR2) can convert A to I in a targeted double stranded RNA at the site of a mismatch between A and C. Moreover, novel evolved ADARs can even act as cytidine deaminase to edit C to U (10). Cas13-based ADAR mediated RNA editing should still prove itself for its therapeutic potential, however, there are uses of ADARs to correct disease causing single nucleotide mutations already. Sinnamon, et al., (12) used an alternative approach to direct ADAR2 to a mutation site on *Methyl CpG Binding Protein 2 (MECP2)* mRNA that causes Rett syndrome, a neurological disease, and efficiently repaired the mutation to reverse its pathological consequences.

PATZ1 is an architectural transcription factor belonging to POZ domain Krüppel-like (POK) zinc finger family. It has multiple roles in variety of biological processes such as cellular proliferation, apoptosis, T-cell differentiation, and embryogenesis (13). PATZ1 inhibits the function of the p53 tumor suppressor protein by binding p53 to modulate its DNA binding capacity (7). Recently, *PATZ1* gene locus is also indicated in various gene fusions including the famous *EWSR1* gene locus that results in the production of chimeric EWSR1::PATZ1 protein that causes round cell sarcoma (15). In order to elucidate the contributions of PATZ1 protein to tumorigenesis and to its other biological roles, utilizing recent molecular biology tools to manipulate PATZ1 expression in mRNA and protein levels would be beneficial.

In the current study, we first designed a gRNA targeting the human *PATZ1* mRNA. This gRNA would guide ADAR2-fused PspCas13b protein to alter the AUG start codon to IUG, which is supposed to inhibit the protein translation initiation from this start codon. Cox, et al., (3) suggests that an effective size of gRNA should be about 50 nucleotide long and the target A should be positioned at around 34th nucleotide. Therefore, with all these variabilities, we also recommend that it is best to design and clone several different guide RNAs surrounding the target nucleotide and test editing efficiencies of these plasmids in an easy-to-transfect human cell line such as HEK293 by co-transfecting them with the plasmid encoding dPsp-Cas13b-ADAR2DD (pC0054) before transitioning to more challenging cell lines or *in vivo* studies. The efficiency of RNA editing at the start codon might be assessed by western blot for the protein production or by qRT-PCR using Taqman probes for the wildtype and mutated start codon on mRNA. The gRNA expressing plasmid we cloned in the current study is 1 nucleotide shorter than the intended sequence. This was probably due to the impurities in the initial oligo synthesis. We had ordered only standard de-salting for the synthesis of our oligos, however, now we recommend that researchers should select for an additional HPLC purification to get the precise length of the oligo before cloning it into the plasmid. *PATZ1* mRNA has other in-frame start codons downstream of canonical start codon that we also targeted in the current study. Therefore, it is important to keep in mind that skipping the initial start codon might not halt the protein translation completely but might yield in N-terminal truncated PATZ1 protein production as well. PATZ1 protein has an N-terminal BTB domain that is important for its activities including dimerization and co-repressor interactions (14). Therefore, skipping the canonical start codon by editing AUG to IUG might be useful for molecular biology studies in various ways.

Conclusion

Here, we report that we designed and cloned a gRNA plasmid that is expected to edit the start codon of human *PATZ1* mRNA when co-transfected with an ADAR2-fused catalytically inactive PspCas13b expressing plasmid into human cells. ADAR2 can edit adenine to inosine with high efficiency and therefore any adenine that is of interest might potentially be edited to inosine with this particular CRISPR/Cas13 approach. Designing and cloning gRNA plasmids are very straightforward and many other gRNAs targeting different RNA sequences may be cloned in a similar way with a limited budget and in a short time frame.

Declarations

Funding

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Conflict of Interest

The authors have declared no conflict of interest.

Ethics Approval

The current design and the execution of this study do not require an ethics approval.

Availability of Data and Material

All data have been presented here. Material may be available upon request.

Authors' Contributions

CG and ED performed the research, analyzed the data, and wrote the paper; ED conceived of and designed the overall study.

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