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Short communication

CRISPR/Cas9-mediated target validation of the splicing inhibitor Pladienolide B

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Abstract

CRISPR/Cas9 system confers molecular immunity in archeal and bacterial species against invading foreign nucleic acids. CRISPR/Cas9 system is used for genome engineering applications across diverse eukaryotic species. In this study, we demonstrate the utility of the CRISPR/Cas9 genome engineering system for drug target validation in human cells. Pladienolide B is a natural macrolide with antitumor activities mediated through the inhibition of pre-mRNA splicing. To validate the spliceosomal target of Pladienolide B, we employed the CRSIPR/Cas9 system to introduce targeted mutations in the subunits of the SF3B complex in the HEK293T cells. Our data reveal that targeted mutagenesis of the SF3b1 subunit exhibited higher levels of resistance to Pladienolide B. Therefore, our data validate the spliceosomal target of Pladienolide B and provide a proof of concept on using the CRISPR/Cas9 system for drug target identification and validation.

Keywords: CRIPSR/Cas9; Pladienolide B; Spliceosome; Pre-mRNA splicing; Drug target validation; Drug discovery

Targeted modification of genomes holds much promise in functional analysis studies in basic biology and applied biotechnology [1,2]. Site-specific nucleases (SSNs) that can be customized to specifically bind and cleave a user-defined sequence have been developed including zinc finger nucleases (ZFNs), transcription activator like nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas9) systems [3–6]. SSNs generate double strand breaks (DSBs) that are repaired by either the error-prone non-homologous end joining (NHEJ) or the precise homology directed repair (HDR), where a template is provided to copy the information across the break [7,8]. Due to its facile engineering and higher efficiencies, CRISPR/Cas9 system is the platform of choice for targeted editing and mutagenesis of eukaryotic genomes [9,10]. Therefore, CRISPR/Cas9 has been used across diverse eukaryotic species for targeted genome editing and regulation and for functional analysis studies [3–5].

Studies of drug target discoveries were hindered by the lack of efficient and reproducible-targeted modification of mammalian genomes. The efficiency of the CRISPR/Cas9 system, and the ability of modifying several targets simultaneously (multiplexing), and amenability of sgRNA library construction and screening are poised to expedite the drug discovery and development efforts for different applications including the treatment of human diseases [5,11–15]. Pladienolide B (PB), a natural product with antitumor activities isolated from *Streptomyces platensis* and shown to bind the SAP130 subunit of the SF3B complex [16,17]. The SF3B complex is part of the U2 snRNP complex [18–20]. Binding of PB to the SF3B complex blocks splicing and prompts

Abbreviations: SSNs, Site Specific Nucleases; ZFN, Zing Finger Nucleases; TALENs, Transcription Like Effector Nucleases; CRISPR, Clustered Regulatory Interspaced Short Palindromic Repeats; Cas9, CRISPR associated protein; sgRNA, Guide RNA; NHJE, Non-Homologous End-Joining; HR, Homologous Recombination; DSB, double strand break; PB, Pladienolide B; AB, Alamar Blue; T7EI, T7 Endonuclease I.

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nuclear export of intron containing transcripts [17,21,22]. Here, we attempted to validate the molecular target of the PB in the SF3B complex. Therefore, we generated sgRNAs with binding specificities to all SF3B subunits. We performed transfections into HEK293T cells using Cas9 endonuclease driven by the β-chicken promoter and sgRNA driven by the U6 promoter (Fig. S1A). We designed several sgRNAs to target some subunits of the spliceosome complex that is including some of SF3B complex such as *SF3B1*, *SF3B2*, *SF3B3* [23] and SF3A complex such as *SF3A3* (Fig. S1B and Supporting information and Table S2). Catalytically inactive Cas9 (dCas9) was used as a negative control (Fig. S1A). Three days post-transfection, cells were collected and genomic DNA purified and subjected to T7 Endonuclease I (T7EI) mutation detection assays. Our data reveal high levels of targeted mutagenesis of the SF3B subunits as demonstrated by T7EI mutation detection assays (Fig. 1A–D). To validate the targeted mutagenesis efficiencies and precisely determine the nature of indels, we PCR-amplified fragments encompassing the target sites, *SF3B1*, *SF3B2*, *SF3B3*, and *SF3A3*, and cloned the amplicons. Subsequently, we subjected these clones to Sanger sequencing and analyzed the presence of indels and the efficiency of targeted mutagenesis (Fig. 1A–D). We achieved 23% of targeted mutagenesis of the *SF3B1* subunit, 37.5% for the *SF3B2*, 35.7% for the *SF3B3* subunit and 20% for the *SF3A3* subunit (Fig. 1E).

Having achieved higher levels of targeted mutagenesis in all subunits of the SF3B complex, we proceeded to investigate the effects of the targeted mutagenesis of each subunit to the sensitivity to PB. Three days post transfection, cells were collected and seeded again in a 24 well culture plate and cultured for 24 h prior treatment with increasing concentrations of PB (1–100 nM) and incubated for 24 h at 37 °C humidified incubator with 5% CO_2. Subsequently, at the end of incubation time the media was removed and we performed cell viability assays using Almar Blue (AB) [24–26]. Using this assay, it is possible to spectrophotometrically measure the cellular proliferation. Resazurin (oxidized form) is blue and non-fluorescent, whereas resorufin (reduced form) is red and highly fluorescent. Therefore, AB reduction is a suitable indicator of the cellular viability [27]. The AB assays were carried out according to manufacturer's instructions. In each experiment, wells containing only the AB solution without cells were also prepared and incubated for 5 h (Fig. 2). HEK293T cells edited in *SF3B1* subunit of the spliceosome complex and treated with 100 nM concentration of PB showed a red color when compared to the cells un-transfected or transfected with only sgRNA or wtCas9 only indicating more resorufin accumulation and high level of resistance to the PB (Fig. 2). Higher cell viability levels (50% ± 8) were detected in population of cells co-transfected with wtCas9 and a sgRNA targeting *SF3B1* compared to lower cell viability levels (23% ± 5) for cells co-transfected with dCas9 and sgRNA targeting *SF3B1* (Fig. 2). However, no significant difference has been observed in HEK293T cells co-transfected with *SF3B2*, *SF3B3* or *SF3A3* indicating that SF3b1 is the

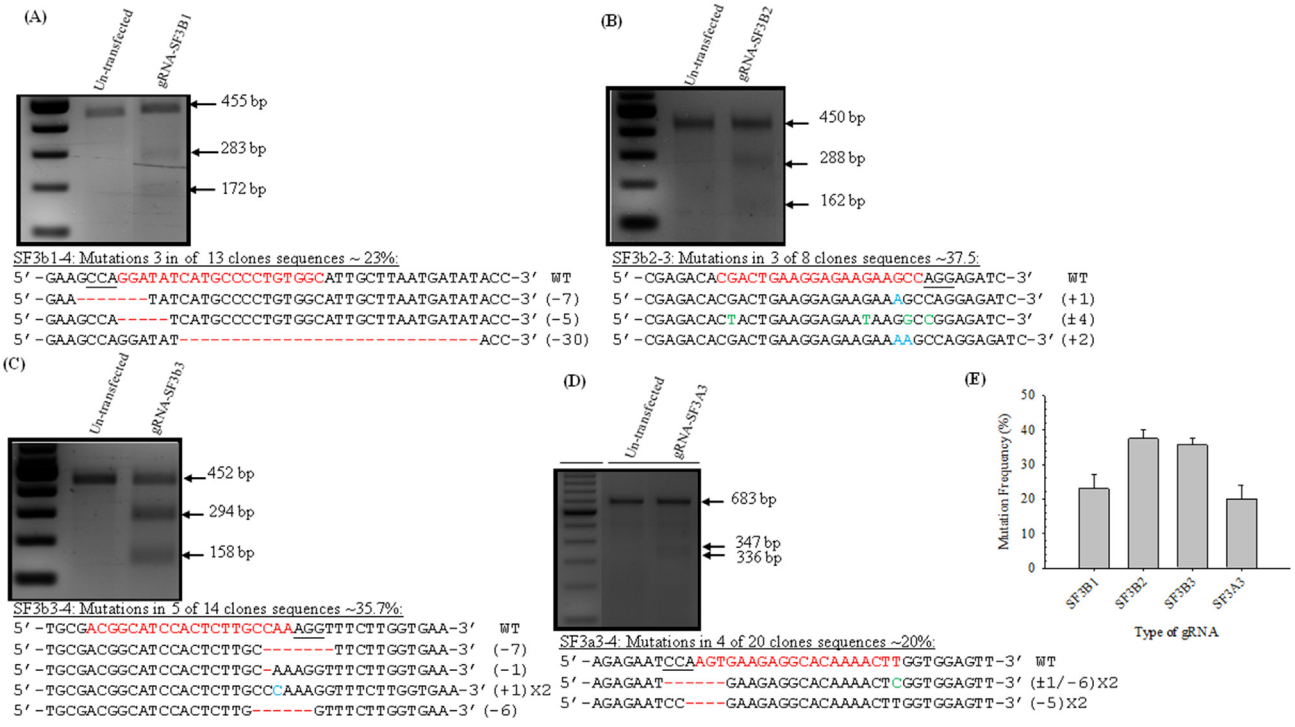

Fig. 1. Detection of the targeted modification of each spliceosome subunit by T7EI assay and Sanger sequencing. (A–D) T7EI assays and alignment of Sanger sequencing reads of PCR amplicons encompassing the targets of *SF3b1*, *SF3b2*, *SF3b3* and *SF3a3* genes respectively. SgRNAs targets are highlighted in red, the PAM sequence is underlined, dashes indicate nucleotide deletions, nucleotides highlighted in blue indicate insertions, and nucleotides highlighted in green indicate substitutions. Arrow indicates the expected size of the DNA bands of corresponding amplicons cleaved by T7EI. (E) Genome editing efficiency in each subunit estimated by the number of mutant clones divided by the total number of sequenced clones.

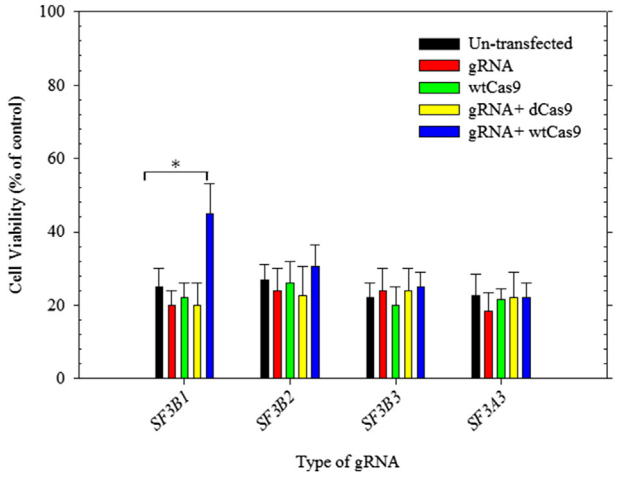

Fig. 2. Effect of the specific gene editing on the toxicity of the antitumor macrolide Pladienolide B on the HEK293T cells. (A) Cell viability was assessed by colorimetric assay using Alamar Blue Cell Viability Reagent (Life Technologies, UK). HEK293T cells edited in various genes (*SF3A3*, *SF3B1*, *SF3B2*, and *SF3B3*) and treated with 100 nM of Pladienolide B for 24 h at 37 °C humidified incubator with 5% CO_2. Specific deletion of *SF3B1* displayed high level of resistance to the PB when compared to the un-transfected cells or cells transfected only with wtCas9, or sgRNA or dCas9. Result is a representative of three independent experiments and the bars represents the mean ± SD and *P < 0.01.

bona fide target of the PB antitumor drug. Our data confirm the conclusions of previous work from Yokoi et al., where a mutation in the gene for *SF3B1* was shown to confer resistance to the inhibitory action of PB [17]. Therefore, CRISPR/Cas9 system could be used to identify a target of known drugs by using a specific subset of sgRNAs and screening their potential targets. Furthermore, sgRNA libraries with genome-wide targets could be employed to generate functional knock outs for the purpose of identifying drug targets (Fig. S2). Our data validate that the SF3B1 is the *bona fide* molecular target the PB and indicate the power of this technology in identifying existing and novel drug targets. Such applications would revolutionize the drug discovery and development efforts, and lead to the next generation of smart and personalized drugs.

Our study extends the utility of the CRISPR/Cas9 system for drug discovery and development applications. Molecular target validation of PB would unlock the possibilities of developing new synthetic analogues that are cheaper and more stable with superb antitumor activities. Furthermore, target knockouts of genes of key cellular machineries, could be used to discover novel drugs from synthetic and natural chemical libraries. Different platforms for genome-wide interrogation of gene function have been recently developed [28]. Such platforms could be utilized to identify the molecular targets of existing drugs, and for novel drug discovery (Fig. S2). Furthermore, such a platform would be helpful in identifying interacting genes and pathways with the primary drug target. By manipulating those interacting genes and pathways, we could increase the efficacy of the current drugs and develop much more powerful drugs. In conclusion, CRISPR/Cas9 system serves as a key tool in the molecular toolbox for drug discovery and development.

Competing interests

Authors declare no competing interests in regards to this project.

Author contribution statement

M.M., M.A., and A.E., designed research, M.A., and A.E., performed research, M.M., and M.A., analyzed data, M.A., prepared the figures, and M.M., and M.A., wrote the manuscript. All authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biopen.2016.02.001.

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