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Hypothesis

Increase the Efficiency of MKN45 Cell Line to CD44 Editing by CRISPR-Cas9: A Hypothesis About P53 Suppression in Gene Editing

Saeid Karimi [1], **Akram Alizadeh** [2], **Nasibe Tabibi** [3], **Sorayya Ghasemi** [1*]

[1] Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[2] Department of Tissue Engineering and Applied Cell Sciences, Faculty of Medicine, Semnan University of Medical Sciences, Semnan, Iran
[3] Department of Medical Genetics, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Corresponding Author: Sorayya Ghasemi, PhD, Associate Professor, Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran. Tel: +98-9131856090, E-mail: s.ghasemi@skums.ac.ir

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Abstract
The clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) used for genome editing. The usage of CRISPR-Cas9 in gene editing is faced with certain limitations including off-target mutation, decreased homologous recombination (HR) repair, and immune system responses. It seems that if Cas9 expressed in an inducible manner, off-target mutations may decrease. The P53 protein decreases the activity of the HR pathway in the cell cycle, so, the decrease in P53 expression level may increase the activity of this pathway. Based on this topic, for the first time, we designed ''px601-Turbo GFP-TRE-shRNA P53'' as a CRISPR-based vector. The use of this vector can simultaneously induce expression of Cas9 and shutdown transiently P53 expression under an inducible promoter and an inducing agent. Therefore, shutdown transiently P53 may be leading to reduced off-targets and increased accuracy of genome editing. In the human gastric cancer MKN45 cell line, the *P53* gene expresses at a normal level. Moreover, CD44 in this cell line has overexpression and is a gastric cancer stem cell marker. To evaluate this hypothesis, CD44 will be targeted for a specific sequence change (editing) by the px601-Turbo GFP-TRE-shRNA P53 vector. Accordingly, after cloning and virus preparation, MKN45 cell lines will be transduced in the presence of the appropriate doxycycline (DOX) dosage. Ultimately, to evaluate the vector efficiency, DNA extraction and whole-genome sequencing (WGS) will be done and compared with the transduced MKN45 cells without an inducible prompter and DOX as control group. Furthermore, the Sanger sequencing for the target gene must be done. This temporary inducible expression of P53 may appear to increase the efficiency of the CD44 gene editing and reduce off-targets.

Keywords: CRISPR–Cas9, P53, HR Repair Pathway, CD44, Off-targets
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Introduction

Gastric Cancer (GC) is one of the most causes of cancer-related fatality. Due to the therapeutic resistance and complexity, GC has to be treated in different approaches.[1] Gene therapy via clustered, regularly interspaced, short palindromic repeats (CRISPRs), and CRISPR-associated protein (Cas9) system is novel with a high potential therapeutic approach in the GC treatment.[2] In this system, as an endonuclease, Cas9 can target DNA sites through guide RNA (sgRNA) and induce Double Stranded Break (DSB) in the desired target DNA. DSBs activate two major types of DNA repair systems: 1) Error-prone Non-Homologous End Joining (NHEJ) causes small insertions and deletions (indels), 2) Homology-Directed Recombination (HDR) leads to genetic replacement by involving exogenous donor DNA sequences.[3] The NHEJ pathway is operated at the G1 phase of the cell cycle. While at the S or G2 phases, the Homologous Recombination (HR) system is activated, and subsequent CDK (cyclin-dependent kinases) activity prevents NHEJ.[4] P53 involvement in the HDR process would be inhibited and neutralize G1 arrest and deactivate the HDR system.[5,6] It has been demonstrated that Cas9-derived DSBs activate P53 and subsequently growth arrest at the G1 phase, which finally increases NHEJ and reduces HDR.[7] Thus, cell cycle arrest at the G1 phase may inhibit efficient HDR by P53 function, and instead, unsuitable repair may occur through NHEJ.[4] In contrast to some GC cell lines, wild-type P53 expresses in MKN45 cells.[8] CD44 is also a Cancer Stem Cell (CSC) marker in the MKN45 cell line that is overexpressed in this cell line. CD44 has an important role in the therapeutic resistance of GC and its associated mechanisms.[9] In view of the above, we suggest with our vector design, in the MKN45 cell line, it may be possible to increase the efficiency of CD44 gene editing by P53 temporarily inhibiting and reducing off-targets via inducible Cas9 expression.

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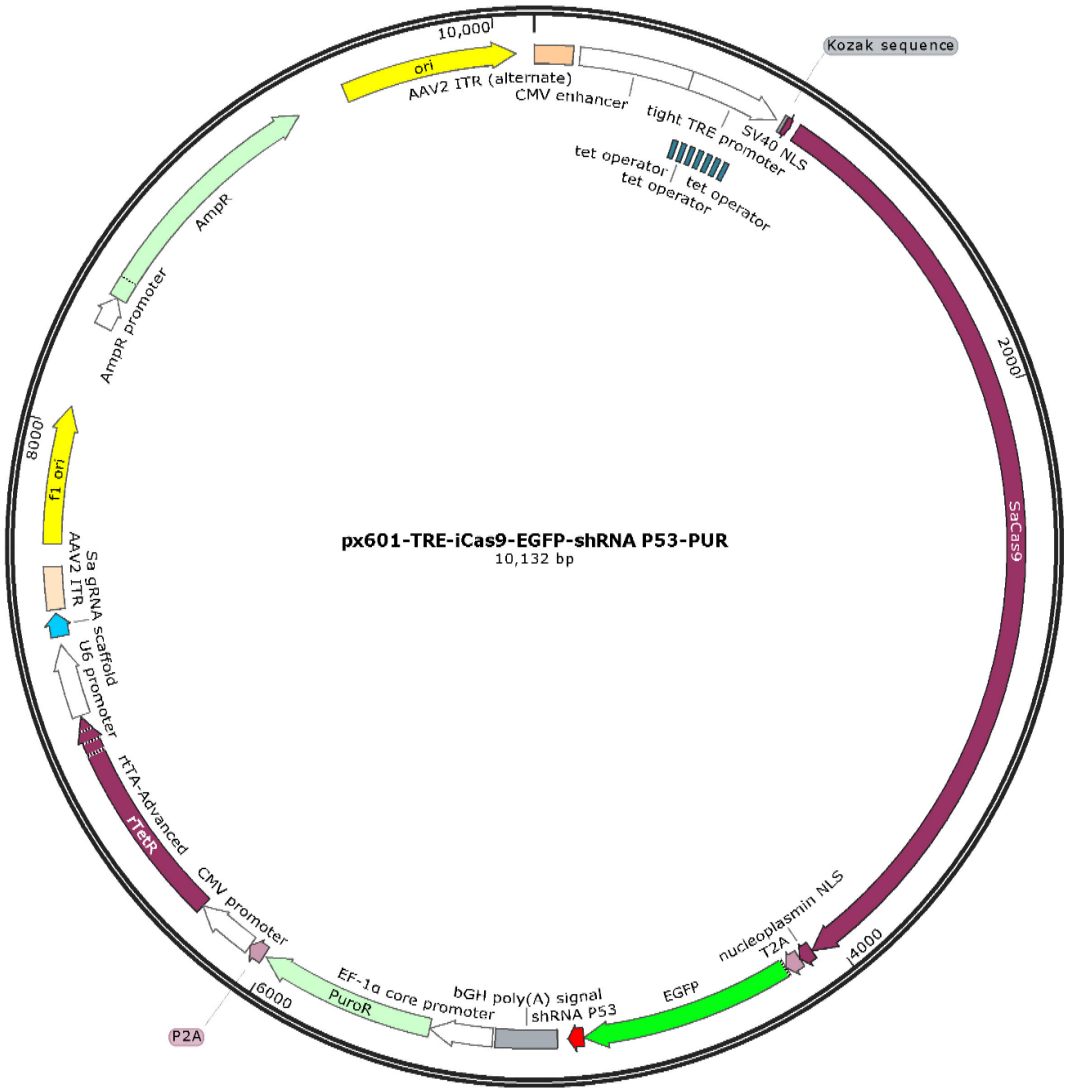

Figure 1. px601-TRE-iCas9-EGFP-shRNA P53-PUR: In the AAV CRISPR vector designation, a type II CRISPR/Cas9 system, *Streptococcus aureus*-derived SpCas9 and P53shRNA sequences were used. This vector contains an inducible tetracycline-dependent promoter (TRE) for the expression of SpCas9 and p53shRNA that are only active in the presence of DOX, while a distinct U6 promoter makes sgRNA expression constant. In addition, the AAV CRISPR vector contains selection markers, including ampicillin, puromycin, and turbo GFP gene. The px601-TRE-iCas9-EGFP-shRNA P53-PUR vector was designed by SnapGene (GSL Biotech, USA) version 3.2.1.

Presentation of the Hypothesis

To investigate the role of transient suppression of P53 protein, we hypothesized a specific designation of Adeno-Associated Virus-CRISPR (AAV-CRISPR). This vector can be used to induce the expression of Cas9, and CD44 sgRNA Cas9-independently, which ultimately leads to a substantial achievement (Figure 1).

Currently, one of the limitations of CRISPR-Cas9 is a **c**onstant Cas9 expression which leads to more off-target mutagenesis. Moreover, Cas9 is an extrinsic antigen triggering host immune response that results in the deletion of the cells from the edited pool, thus affecting the efficiency and precision of the CRISPR-Cas9 tool for editing the genome.[10] Moreover, as shown in Figure 2, P53 expression involvement in the HDR process would be inhibited, leading to neutralizing G1 arrest and activating the HDR system.[5,6] Many studies have demonstrated Cas9-derived DSBs activate P53 and subsequently growth arrest at the G1 phase, which finally increases NHEJ and reduces HDR. This is a challenge for genome editing applications. HDR is less usual than NHEJ and arises only during S and G2 phases, whereas NHEJ arises throughout the cell cycle. HDR occurs not basically but rather simultaneously with NHEJ, and is heightened in NHEJ defective cells.[11] Van Trung Chu et al., showed an effective way to authorize accurately targeted mutations into the genome of mammalian cells by suppressing the NHEJ key enzyme DNA ligase IV that will lead to gene corrections. Furthermore, the NHEJ pathway can be suppressed by inhibition of P53.[12] Even though the P53 expression inhibition makes the cell vulnerable to chromosomal rearrangements

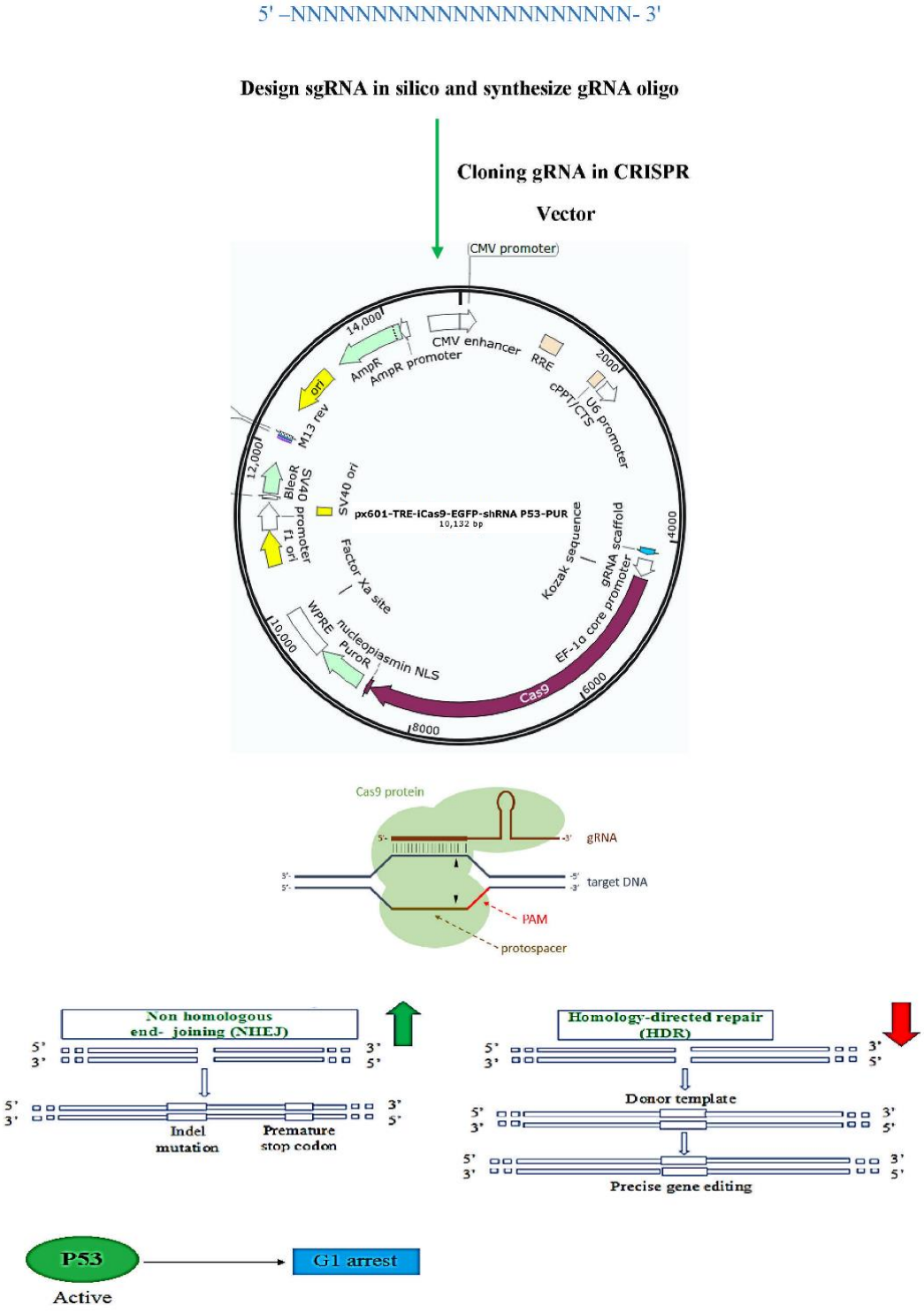

Figure 2. px601-TRE-iCas9-EGFP-shRNA P53-PUR; Inducible Cas9 and shRNA P53 by AAV CRISPR vector enhance the HDR repair and reduce NHEJ repair compared to other CRISPR vectors, which could improve the accuracy and function of the CRISPR-Cas9 system. shRNA P53 sequence: GACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACACTGGAGTCTTTTT.

and other tumorigenic mutations, some studies have showed no harmful cellular alteration such as carcinogenesis in case of short-term inhibition of P53.[10,13] Furthermore, using even inducible P53 inhibition improves the long-term survival of mice.[14] The inducible promoter (TRE) of Cas9 and shRNA P53 is capable of induction with a specific inducer for TRE for a limited time.

It has been shown that the transgenic mice with inducible CRISPR-Cas9 edition by DOX treatment were successfully cloned.[10] Therefore, it seems that the administration of DOX at lower concentrations, commonly used with inducible expression systems, had no effect on cell growth.[13]

Testing the Hypothesis

CD44 RNA guide sequence will be designed, synthesized, and cloned into this vector. Then, the packaging will provide particle viruses and be transduced into the MKN45 cell line. DOX at lower concentrations (10 μg/ml) can be used to induce expression of P53 and Cas9 independently and synchronic. Then, FACS will be used to detect correcting GFP, and the RNP-transfected MKN45 cells will be cultured. After four days, recombinant clones will be selected by puromycin or any marker located in the vector. MKN-45 cell line transduced without an inducible prompter and DOX) will be a control group. Ultimately, for efficiently evaluating of the px601-Turbo GFP-TRE-shRNA P53 vector, the cellular DNA of the cell groups will be extracted separately. Then, the Whole Genome Sequencing (WGS) will be done for the cell groups. The data will be analyzed and compared to investigate whether the vector can lead to precise and improved genome editing without side effects (off-targets) in the cells. As well as, the Sanger sequencing for the target gene must be done. The rate of marker expression correction in the two group cells can be compared by Real-Time PCR. *In vivo* studies in animal models can also be made. The steps are similar to *in vitro* approach.

Implications of the Hypothesis

Although CRISPR-Cas9 is known as one of the best tools for editing genomes, it still has some limitations, including immune responses to the constant expression of Cas9, induction of a P53-mediated DNA damage response, and cell cycle arrest at the G1 phase, resulting in activation of NHEJ rather than HDR.[10,14,15] The vector proposed in this study probably can overcome the limitations through an inductive expression of Cas9 and temporarily inhibit P53. Thus, this vector may improve the CRISPR-Cas9 efficiency in genome editing and the outcomes of this technology. We expect the level of CD44 expression to be different in the two groups and the rate of expression change to be closer to normal in the group with induced expression.

Conclusion

The precise genome editing using CRISPR–Cas9 is based on recruiting HDR, most active at the S phase of the cell cycle. Thus, cell cycle arrest at the G1 phase may inhibit efficient HDR by P53 function, and instead, improper repair may occur through NHEJ. Moreover, recent evidence has demonstrated the crucial role of P53 in cell resistance to CRISPR-Cas9 gene editing via HR repair. This vector improves the efficiency of CRISPR-Cas9 in editing genomes and reduces off-targets and cell resistance in using the HR repair system. Thus, we hypothesized and suggested the "px601-Turbo GFP-TRE-shRNA P53" vector to reduce off-targets, immune responses, and cell resistance effectively.

Authors' Contributions

SK done initial design hypothesis, draw vector map, and provided images and drafted the manuscript. NT and AA searched and contributed to the writing. SGH was a major contributor in the design of this hypothesis and writing and revising the manuscript.

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Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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References

1. Moradzadeh R, Anoushirvani AA. Trend of gastric cancer incidence in an area located in the center of Iran: 2009–2014. J Gastrointest Cancer. 2020;51(1):159-64. doi:10.1007/s12029-019-00227-8
2. Kanda T, Furuse Y, Oshitani H, Kiyono T. Highly efficient CRISPR/Cas9-mediated cloning and functional characterization of gastric cancer-derived Epstein-Barr virus strains. J Virol. 2016;90(9):4383-93. doi:10.1128/JVI.00060-16
3. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. Nat Med. 2015;21(2):121-31. doi:10.1038/nm.3793
4. Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol. 2008;9(4):297-308. doi:10.1038/nrm2351
5. Kelly RM, Goren EM, Taylor PA, Mueller SN, Stefanski HE, Osborn MJ, et al. Short-term inhibition of p53 combined with keratinocyte growth factor improves thymic epithelial cell recovery and enhances T-cell reconstitution after murine bone marrow transplantation. Blood. 2010;115(5):1088-97. doi:10.1182/blood-2009-05-223198
6. Lee CL, Castle KD, Moding EJ, Blum JM, Williams N, Luo L, et al. Acute DNA damage activates the tumour suppressor p53 to promote radiation-induced lymphoma. Nat Commun. 2015;6(1):8477. doi:10.1038/ncomms9477
7. Haapaniemi E, Botla S, Persson J, Schmierer B, Taipale J. CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. Nat Med. 2018;24(7):927-30. doi:10.1038/s41591-018-0049-z
8. Ji W, Ma J, Zhang H, Zhong H, Li L, Ding N, et al. Role of p53β in the inhibition of proliferation of gastric cancer cells expressing wild-type or mutated p53. Mol Med Rep. 2015;12(1):691-5. doi:10.3892/mmr.2015.3370
9. Terzioğlu G, Turksoy O, Bayrak OF. Identification of An mtDNA setpoint associated with highest levels of CD44 positivity and chemoresistance in HGC-27 and MKN-45 gastric cancer cell lines. Cell J (Yakhteh). 2018;20(3):312-7. doi:10.22074/cellj.2018.5309
10. Chew WL, Tabebordbar M, Cheng JK, Mali P, Wu EY, Ng AH, et al. A multifunctional AAV–CRISPR–Cas9 and its host response. Nat Methods. 2016;13(10):868-74. doi:10.1038/nmeth.3993
11. Maruyama T, Dougan SK, Truttmann M, Bilate AM,

Ingram JR, Ploegh HL. Inhibition of non-homologous end joining increases the efficiency of CRISPR/Cas9-mediated precise [TM: inserted] genome editing. Nat Biotechnol. 2015;33(5):538-42. doi:10.1038/nbt.3190
12. Van Chu T, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, et al. Erratum: increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol. 2018;36(2):196. doi:10.1038/nbt0218-196d
13. Katigbak A, Robert F, Paquet M, Pelletier J. Inducible genome editing with conditional CRISPR/Cas9 mice. G3-GENES GENOM GENET. 2018;8(5):1627-35. doi:10.1534/g3.117.300327
14. Luger AL, Sauer B, Lorenz NI, Engel AL, Braun Y, Voss M, et al. Doxycycline impairs mitochondrial function and protects human glioma cells from hypoxia-induced cell death: implications of using tet-inducible systems. Int J Mol Sci. 2018;19(5):1504. doi:10.3390/ijms19051504
15. Ihry RJ, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, et al. p53 inhibits CRISPR–Cas9 engineering in human pluripotent stem cells. Nat Med. 2018;24(7):939-46. doi:10.1038/s41591-018-0050-6