A CRISPR Platform for Rapid and Inducible Genome Editing in Human Non-small Cell Lung Cancer Cells

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Introduction

Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancer, which is the leading cause of cancer death in the world. High mortality rate associated with NSCLC is partially attributed to the limited understanding of NSCLC as well as ineffective therapeutic treatments. The initiation and progression of NSCLC involves genetic changes leading to alterations in the control of tissue development and homeostatic maintenance. Better knowledge about these genetic abnormalities is imperative for developing new chemotherapeutic drugs for NSCLC. To understand the biological functions of genetic mutations in NSCLC cells, it is important to generate a platform that efficiently modulates expression of genes implicated in the development of NSCLC. Recently, several gene-editing systems have been developed to modify genomes in a variety of model systems. Among which, transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems have emerged as effective experimental tools to achieve this goal. In several studies of human pluripotent stem cells, TALENs and CRISPR/Cas have been combined to achieve rapid and controllable genome modulations.

Objectives

The goal of this study is to establish an efficient and scalable experimental system to interrupt gene expression in NSCLC cells by combining TALENs and CRISPR/Cas9 nucleases. This strategy will provide a platform to investigate how changes in gene expression (e.g. PON2) are involved in the initiation and development of NSCLC.

Results

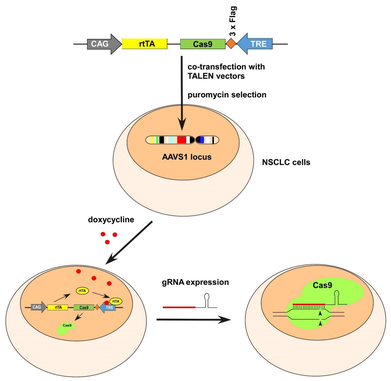

Figure 1. A platform for efficient and inducible genome editing in human NSCLC cells.
A genome editing platform was generated in human NSCLC cells by combining TALENs and CRISPR/Cas9 nuclease systems. A vector expressing Cas9 from an inducible promoter with tetracycline response element (TRE) and a pair of TALEN vectors targeting the transcriptionally active AAVS1 locus were co-transfected into the NSCLC cell line NCI-H1299. Stable clones were acquired after culturing the cells in the medium containing puromycin. The expression of Cas9 protein (green) was induced by doxycycline (red dots). Cas9 proteins associate with exogenously expressed gRNA containing a specific DNA recognition sequence (red). The Cas9/gRNA complex binds to DNA and induces a double strand break (arrow heads) at an specific DNA site, leading to genome editing. CAG, constitutive synthetic promoter; rtTA, reverse tetracycline transactivator sequence and protein.

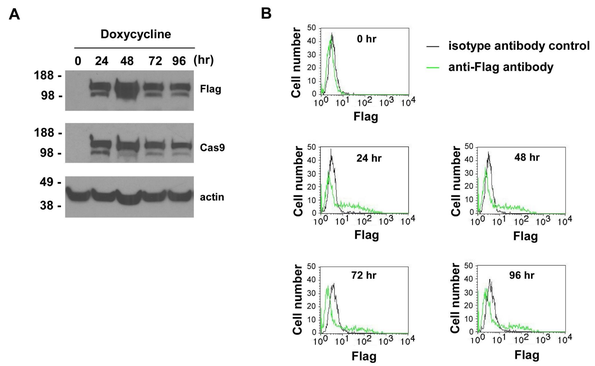

Figure 2. Cas9 expression is induced by doxycycline in NSCLC cells.
(A) NCI-H1299 cells were transfected with a vector expressing Cas9 from a doxycycline-inducible promoter and two AAVS1 locus-targeting TALEN vectors. Stably transfected cells were acquired after culturing with puromycin in the medium. Cas9 expression in the bulk population of stably transfected cells was examined by western blot after culturing with12 μM doxycycline for the indicated time. Actin served as a loading control. Molecular weight markers are shown on the left.
(B) Immunofluorescent staining experiments were carried out to evaluate the induction of Cas9 expression in NCI-H1299 cells. The bulk population of stably transfected NCI-H1299 cells were cultured with 12 μM doxycycline for the indicated time. Cas9 expression was evaluated by immunofluorescent staining using anti-Flag antibodies as primary antibodies and Alexa-Fluor-488 anti-mouse antibodies as secondary antibodies. The fluorescent intensity of the cells was evaluated by flow cytometry. The spectrum of fluorescent intensity indicates the diverse expression levels of Cas9 in the bulk population of stably transfected cells.

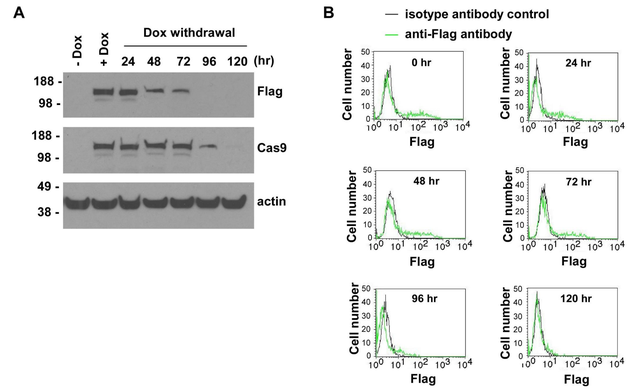

Figure 3. The expression of Cas9 induced by doxycycline is reversible.
(A) The bulk population of NCI-H1299 cells stably expressing Cas9 was cultured with 12 μM doxycycline for 72 hours, and doxycycline withdrawal was carried out by replacing with doxycycline-free medium. Cas9 expression was examined by western blot at the indicated time points. Actin was detected as a loading control. Molecular weight markers are indicated on the left. Dox, doxycycline.
(B) After culturing with 12 μM doxycycline for 72 hours, doxycycline was withdrawn from the medium of transfected NCI-H1299 bulk population for the indicated periods of time. Immunofluorescent staining was applied to examine Cas9 expression with anti-Flag antibodies serving as primary antibodies and Alexa-Fluor-488 anti-mouse antibodies functioning as secondary antibodies. The fluorescent intensity of the cells was determined using flow cytometry. The time points of doxycycline withdrawal are indicated in each panel.

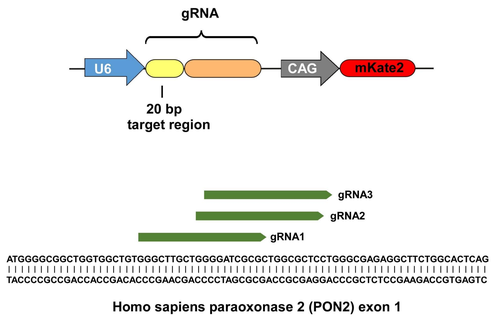

Figure 4. Expression of gRNAs targeting the first exon of human PON2 gene.
Recent research demonstrates that the expression of paraoxonase 2 (PON2), a lactonase/arylesterase with anti-oxidant properties, are markedly enhanced in cancer tissues of NSCLC patients and increased PON2 expression likely contributes to the resistance of NSCLC cells to classical anti-NSCLC therapeutic drugs. The sequence of three gRNAs targeting the first exon of human PON2 was acquired from Optimized CRISPR Design (MIT) and cloned into the vector gRNA-CKB. The schematic diagram of gRNA-CKB is illustrated, in which gRNAs are expressed from a U6 promoter and the red fluorescence-emitting marker protein mKate2 is expressed from a CAG promoter.

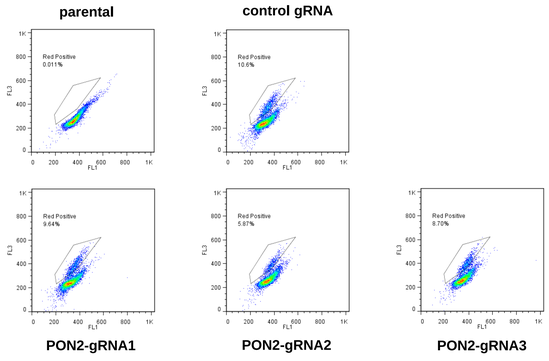

Figure 5. gRNAs specific for human PON2 gene are expressed in NSCLC cells.
Clones of NCI-H1299 cells expressing inducible Cas9 were acquired by flow cytometry sorting. The gRNA-CKB vectors encoding control gRNA or gRNAs specific for the first exon of human PON2 gene were transfected into Cas9-expressing NCI-H1299 cells using lipofectamine™ 2000. Stably transfected cells were acquired by culturing them in the medium containing blasticidin. The expression of the marker protein mKate2 in the bulk population of transfected cells was examined by flow cytometry seven days after transfection and the data were analyzed by the software FlowJo. The percentage of cells positive for red fluorescence (mKate2) is shown in each graph.

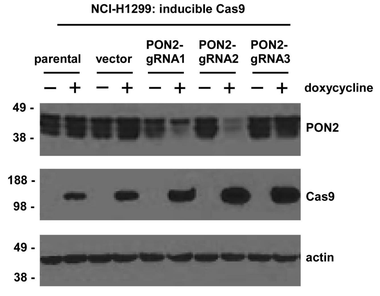

Figure 6. Doxycycline induces a reduction of PON2 expression in NSCLC cells expressing inducible Cas9.
Inducible Cas9-expressing NCI-H1299 cells were stably transfected with the vectors containing control gRNA or gRNAs targeting human PON2 gene, and the cells expressing gRNAs were acquired by flow cytometry sorting of the marker protein mKate2. The expression of Pon2 and Cas9 in the bulk population of sorted cells was examined by western blot after culturing with12 μM doxycycline for 96 hours. Actin served as a loading control. Molecular weight markers are shown on the left.

Conclusion

Human NSCLC cells stably expressing inducible Cas9 have been generated. The expression of Cas9 in NSCLC cells is reversible. Doxycycline induces a reduction of PON2 expression in NSCLC cells expressing inducible Cas9 and gRNAs specific for PON2 gene. This study provides a foundation for a platform of efficient genome editing in NSCLC cells.

Future studies

gRNAs specifically targeting the genes involved in NSCLC initiation and development will be expressed in NSCLC cells with inducible Cas9. The mechanisms of how these genes modulate cell proliferation and resistance to therapeutic drugs of NSCLC cells will be investigated.

Acknowledgements

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