Development of CRISPR Tool Cell Lines for Targeted Gene Expression Study

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Abstract

Developing technologies for the targeted disruption of gene expression will provide powerful tools for studying gene function. To date, various methods for achieving loss-of-function (LOF) outcomes have been developed, including approaches employing RNA interference (RNAi) and CRISPR (clustered regularly interspaced short palindrome repeats)/Cas9-mediated gene perturbation.[1] In mammalian cells, RNAi is the predominant method for programmed knockdown of mRNAs, but its utility is limited by confounding off-target effects.[2] The RNA-guided CRISPR-associated nuclease Cas9 provides an effective means of introducing targeted LOF mutations in the genome. Cas9 can be programmed to induce DNA double strand breaks (DSBs) at specific genomic loci through guide RNAs (gRNA), which when targeted to coding regions of genes can create frame shift indel mutations resulting in a LOF allele.[3] In addition to the use of the nuclease activity of Cas9, the CRISPR-Cas9 technology can also be repurposed as a sequence-specific, non-mutagenic gene regulation tool. Coupling of the engineered nuclease-deficient Cas9 (dCas9) to a transcriptional repressor domain can robustly silence expression of endogenous genes with high specificity, resulting in 'CRISPR interference' (CRISPRi).[4,5] Here we report the creation of Cas9-expressing HEK-293 and CRISPRi A549 cell lines, in which the Cas9 or KRAB-dCas9 expression cassette was integrated into AAVS1 safe harbor locus. The integration of knock-in allele was confirmed at the genomic and translational levels in both cell lines. When gRNAs targeting p53 and RFP genes were delivered into HEK-293 Cas9 cells, DNA double-stranded breaks at intended sites were detected using T7E1 assay and Sanger sequencing, and the expression of p53 and RFP proteins was significantly disrupted. In A549 CRISPRi cells, gRNAs targeting p53 and SETD9 promoter regions repressed p53 and SETD9 gene transcription approximately 75% and 65%, respectively. In p53 gRNA expressing virus infected A549 CRISPRi single cell clones, approximately 95% p53 transcription repression was detected. Taken together, our data suggest that these CRISPR tool cell lines are valuable tools that greatly simplify the study of human gene function and provide potential applications for precise gene knockout and knockdown in human cells.

Results

I. Generation of Cas9 and KRAB-dCas9 Expression Knock-In Allele at AAVS1 Locus

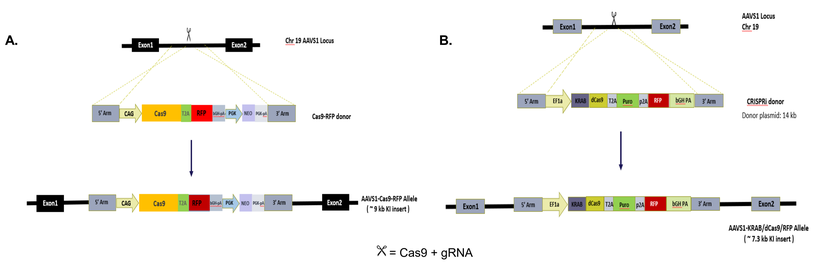

Figure 1. **Gene-editing design for the generation of AAVS1 Cas9 knock-in allele in HEK-293 cells and AAVS1 KRAB-dCas9 knock-in allele in A549 cells.** HEK-293 and A549 parental cells were transfected with AAVS1 gRNA and knock-in donor, along with the Cas9-expressing plasmid. Transfected pooled cells were used for single cell sorting and expanded single cell clones were then subjected to genotyping to identify (A)Cas9-RFP and (B) KRAB-dCas9-RFP knock-in clones. In this design, the RFP and antibiotic selection marker were used for easy detection and selection of knock-in clones.

II. Identification of AAVS1 Cas9 Knock-in Allele and Confirmation of Cas9 Protein Expression in HEK-293 Cells

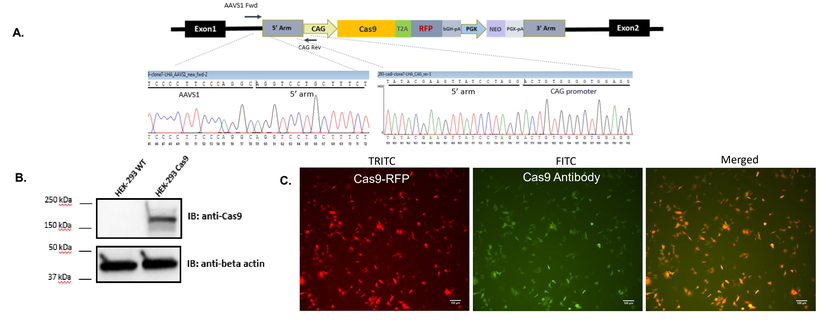

Figure 2. **Identification of Cas9 knock-in HEK-293-Cas9 single cell clones.** (A) Genomic DNA was extracted from expanded single clones for the left homology arm (LHA) junction PCR. Junction PCR primers and their positions are displayed in the diagram. PCR amplicons were subjected to Sanger sequencing. Cas9 knock-in HEK-293 clones exhibited the expected sequences at the LHA junction regions. (B) Cas9 protein expression was detected by western blotting in HEK-293-Cas9 cells but not in HEK-293 parental cells; actin was used as a loading control. (C) Immunocytochemistry (ICC) was performed using Cas9 antibody on HEK-293 Cas9 cells (middle). Cas9 positive cells (middle, green) also express RFP (left, red), as shown in the merged image (right, yellow), further confirming the correct knock-in of AAVS1-Cas9 allele.

III. Identification of AAVS1 KRAB-dCas9 Knock-in Allele and Confirmation of KRAB-dCas9 Protein Expression in A549 cells

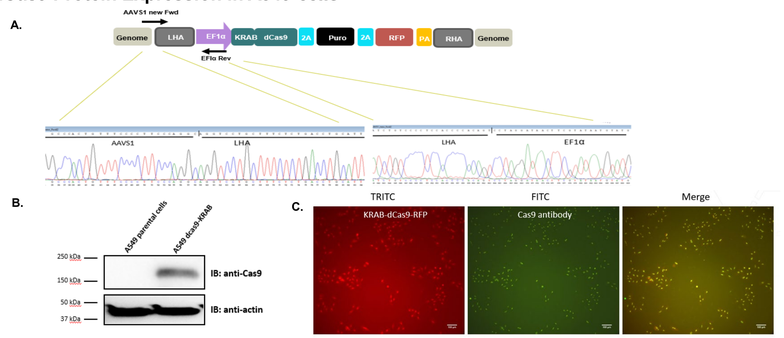

Figure 3. **Identification of AAVS1-KRAB-dCas9 knock-in A549 single cell clones.** (A) Genomic DNA was extracted from expanded single clones for the left homology arm (LHA) junction PCR. Junction PCR primers and their positions are displayed in the diagram. PCR amplicons were subjected to Sanger sequencing. A549 dCas9-KRAB knock-in clones display the expected sequences at LHA junction regions. (B) Cas9 protein expression was detected by western blotting in A549 KRAB-dCas9 cells, but not in A549 parental cells; actin was used as a loading control. (C) ICC was performed using Cas9 antibody on A549 KRAB-dCas9 cells (middle). Cas9 positive cells (middle, green) also express RFP (signal due to functional protein expression; left, red), as shown in the merged image (right, yellow), further confirming the correct knock-in of AAVS1-KRAB-dCas9 allele.

IV. Growth Kinetics and Morphology of HEK-293 Cas9 Knock-in Cells

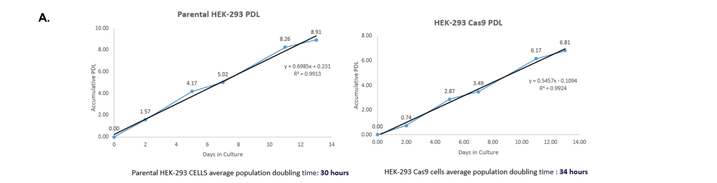

Figure 4. **Growth properties and morphology of HEK-293 Cas9 cells are similar to that of parental HEK-293 cells.** (A) Population doubling time of HEK-293 parental cells and HEK-293 Cas9 cells was measured over 14 days. Comparable growth curves indicated the growth kinetics of HEK-293 Cas9 cells were similar to that of parental HEK-293 cells. (B) HEK-293 parental cells and HEK-293 Cas9 cells displayed similar morphology.

V. Growth Kinetics and Morphology of A549 KRAB-dCas9 Knock-in Cells

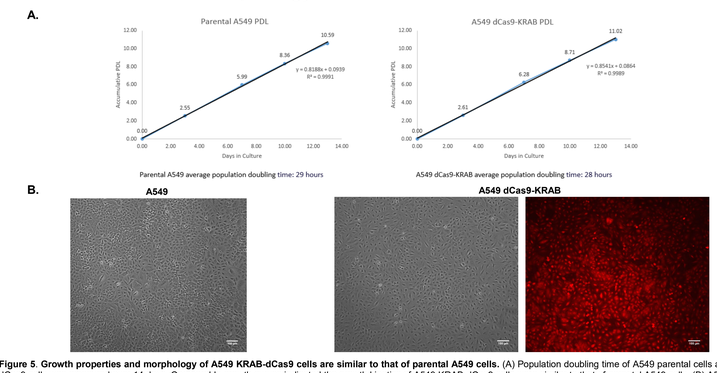

Figure 5. **Growth properties and morphology of A549 KRAB-dCas9 cells are similar to that of parental A549 cells.** (A) Population doubling time of A549 parental cells and A549 KRAB-dCas9 cells was measured over 14 days. Comparable growth curves indicated the growth kinetics of A549 KRAB-dCas9 cells were similar to that of parental A549 cells. (B) A549 parental cells and A549 KRAB-dCas9 cells displayed similar morphology.

VI. Confirmation of Cas9 Cleavage Activity in HEK-293 Cas9 Knock-in Cells

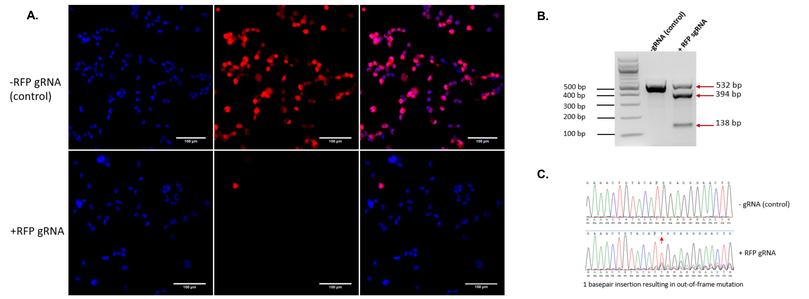

Figure 6. **Confirmation of Cas9 cleavage activity in HEK-293 Cas9 cells.** (A) HEK-293 Cas9 cells were transduced with lentiviruses that do not express gRNA (top panel, as a no gRNA control) or express gRNA targeting RFP (bottom panel, gRNA expressing cells). The nuclei of cells were counterstained with DAPI (blue, left panel), RFP signal (red, middle panel) was the result of functional protein expression; and merged image (right panel). 6 days after transduction, RFP expression in gRNA expressing HEK-293 Cas9 cells was significantly reduced compared to that of control cells. (B) T7 Endonuclease I (T7E1) mismatch assay showed the expected DNA cleavage patterns in gRNA expressing HEK-293 Cas9 cells, but not in control cells. (C) Sanger sequencing of amplicons from figure (B) revealed that a 1 bp insertion (indicated by red arrow) was introduced into the RFP gene in gRNA expressing HEK-293 Cas9 cells, resulting in an out-of-frame mutation of the RFP gene.

VII. Repression of Gene Transcription in A549 dCas9-KRAB Cells

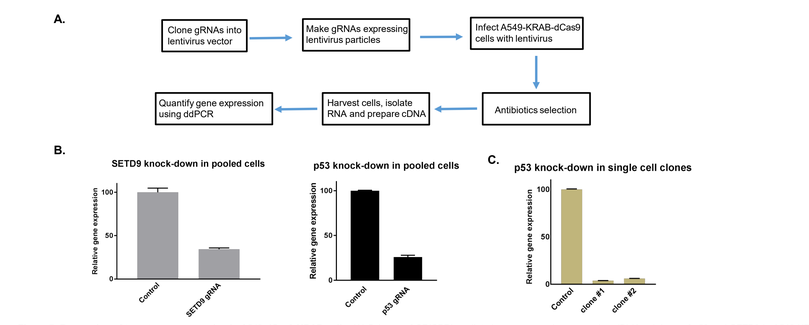

Figure 7. **Repression of target gene expression in A549 dCas9-KRAB cells.** (A) Scheme of CRISPRi-mediated gene expression repression. (B) Knockdown of p53 and SETD9 in A549 KRAB-dCas9 cells. Lentivirus expressing gRNAs targeting p53 gene and SETD9 gene were used individually to infect A549 dCas9-KRAB cells. Lentivirus without gRNA expression was used as the control. 24 hours after infection, antibiotics were added to the culture media to enrich antibiotic resistant cells. Cell pellets were collected after 5 days of selection and subjected to ddPCR gene expression quantification analysis. The expression of SETD9 gene (left), and p53 gene (right) was significantly repressed in cells infected with gRNAs. (C) p53 knock-down stable cell lines were established by isolating antibiotic resistant single cell clones. In two randomly picked cell clones, about 95% reduction of p53 gene expression was detected.

Summary

- The HEK-293 Cas9 and A549 dCas9-KRAB cell line models were successfully created using CRISPR gene-editing technology.
- Both Cas9 and dCas9-KRAB expression cell lines have been extensively validated at the genomic and translational level.
- HEK-293 Cas9 cell line, which stably expresses Cas9 protein, showed high efficiency of targeted DNA cleavage, leading to a corresponding loss of protein expression when gRNAs are delivered.
- A549 dCas9-KRAB cell line, which stably expresses dCas9-KRAB protein, showed > 50% target gene knock-down in pooled cells and > 90% target gene knock-down in a single cell expressing gRNA.
- Our findings demonstrate that HEK-293 Cas9 and A549 dCas9-KRAB cell lines are valuable tools for studying targeted gene expression.

References

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