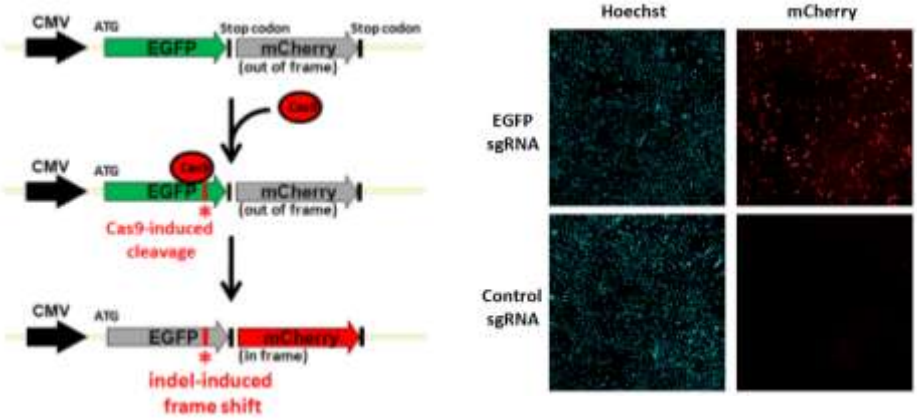
CRISPR Technique Supports

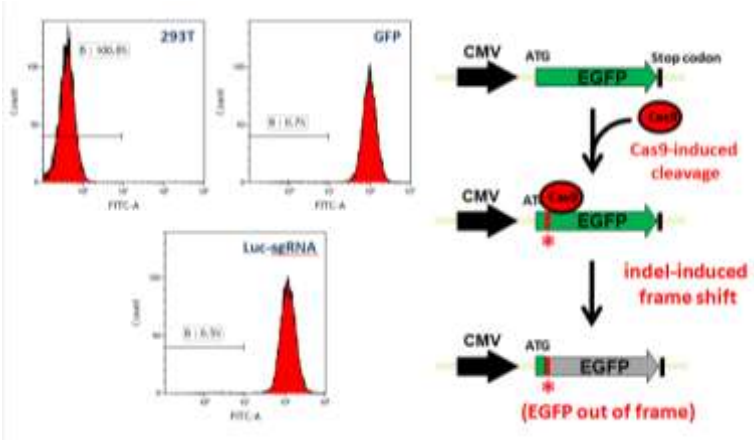
Part Ⅰ: CRISPR-induced indel

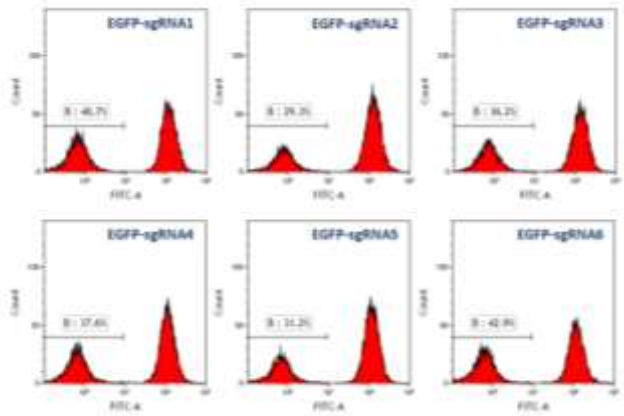
The CRISPR/Cas9 technology is an emerging genome engineering tool and can be readily targeted to specific gene of interest. Taking advantage of site-specific cleavage of the targeted DNA, researcher can easily generate indel within the gene of interest and disrupt its function and/or expression. To visualize the CRISPR-induced indel, a reporter assay was carried out by using a dual-fluorescence reporter, of which the second fluorescent gene (mCherry) was designed to be out-of-frame to the coding sequence of the first fluorescent gene (EGFP). HEK-293T cells were co-transfected with the dual-fluorescence reporter and an all-in-one CRISPR system which targets to the EGFP CDS. Once the Cas9 endonuclease mediated double strand break of the targeted EGFP gene, insertions or deletions (indels) would be introduced by nonhomologous end joining (NHEJ) cellular repair mechanism. The indels may lead to frameshift mutations at the targeted site and thus enable the expression of the downstream mCherry gene (becoming in-frame after indel generation). As evidenced by the appearance of mCherry fluorescence, the NHEJ activity (CRISPR-induced indel) was detected after EGFP sgRNA expression, but not a control sgRNA.



To further confirm that CRISPR-induced indel can efficiently cause loss-of-function of the targeted gene, HEK-293T cells stably expressing EGFP were established as a reporter system and infected with individual lentivirus carrying all-in-one CRISPR to disrupt the expression of EGFP gene. Twenty days after lentiviral transduction, transduced cells were collected to assess the proportions of EGFP(-) and EGFP(+) cells by flow cytometry analysis. All six EGFP-targeting sgRNAs were found to efficiently “knock-out” EGFP expression, as evidenced by raising a significant proportion of EGFP(-) cells,

ranging from 29.3% to 42.9%. On the other hand, Luc-targeting sgRNA did not show any significant change of EGFP(+) proportion as compared to the mock control cells (GFP). Similar proportions of EGFP(-) cells in these sgRNA-targeted cells were observed after culturing these cells for a longer period of time (up to 40 days), indicating that the genomes of these cells were stably altered by CRISPR-induced indels. It should be noted that the genome-integrated CRISPR-cassette (generated by lentivirus transduction) would constitutively express sgRNA and Cas9 protein, which work together to continuously cleave the sgRNA-targeting site(s) until the targeting site was altered. As a consequence, most of cells would carry indels after long-term of CRISPR treatment and of which a significant proportion of cells would show loss-of-function phenotype.

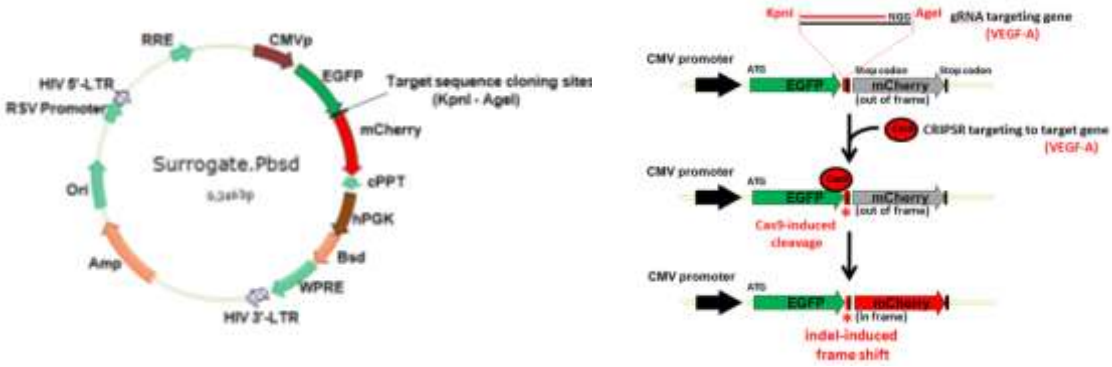




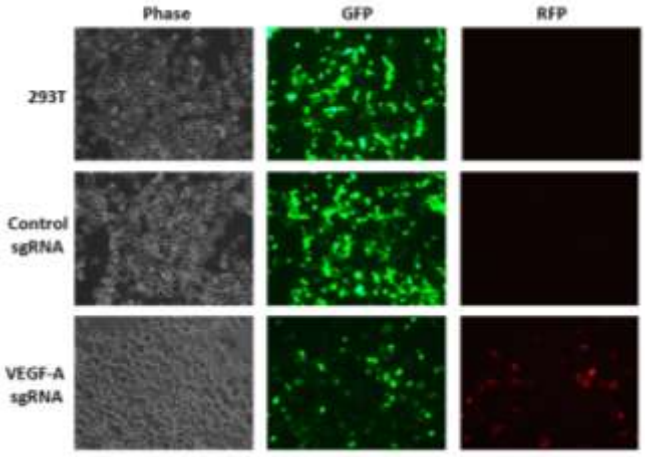
Part Ⅱ: Surrogate reporter assay

It has been well demonstrated that a surrogate reporter construct could be used to present or enrich the cells, wherein the sgRNA-targeting gene is recognized and cleaved by CRISPR/Cas9 system. Here, we constructed a dual-reporter surrogate for examination of NHEJ activity mediated by CRISPR.

As shown below, a linker with restriction enzyme recognition sites (*Kpn*I-*Pme*I-*Afe*I-*Age*I) was introduced in between the EGFP and mCherry genes, which provides multiple cloning sites for cloning of sgRNA-targeting sequence. To examine the efficiency of a given sgRNA and mimic its activity on cleavage of endogenous gene (eg., VEGF-A), the sgRNA recognition sequence including PAM shall be cloned into the linker region.

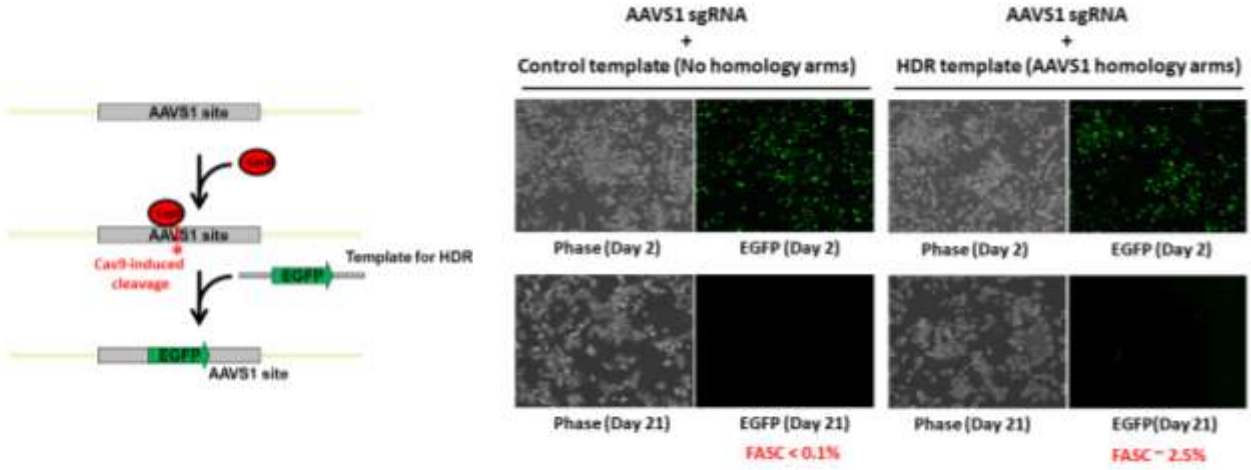


HEK-293T cells were transiently co-transfected with a VEGF-A surrogate reporter and an all-in-one CRISPR plasmid (with VEGF-A sgRNA or control sgRNA expression). Cells were collected two days post-transfection and the expression level of mCherry was detected by fluorescent microscope. Cells carrying VEGF-A surrogate reporter will show signal (indel formation) if the surrogate reporter was recognized by VEGF-A sgRNA and cleaved by Cas9 protein. Indeed, we observed cells carrying red fluorescence as shown in the figure below. T7EI assay also confirmed that indels were generated in the endogenous VEGF-A gene (the sgRNA-targeting site). The result suggests that surrogate reporter could be a useful tool to examine whether delivered sgRNA is functional or not.



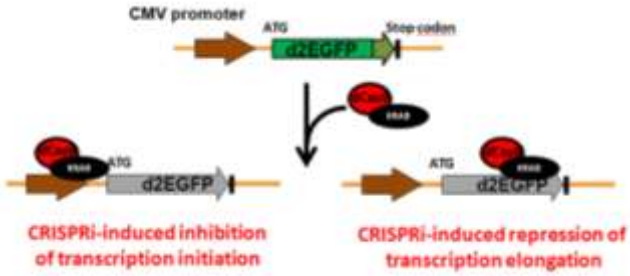
Part Ⅲ: CRISPR-mediated HDR

In addition to NHEJ, CRISPR would also introduce homology directed repair (HDR) if there is a DNA template provided. After creating a nick or a double-strand break (DSB) at sgRNA-targeting site by CRISPR/Cas9, cellular HDR machinery would repair the broken DNA by using a provided DNA template with two homology arms. Taken AAVS1 site as example, the schematic representation (see figure below) shows Cas9 specifically cleaves the sgRNA-targeting AAVS1 site and thus induces HDR occurrence. HEK-293T cells were transiently co-transfected with an all-in-one CRISPR plasmid (expressing a sgRNA targeting AAVS1 site) and an AAVS1 HDR template or a control template. The expression of EGFP was monitored under a fluorescent microscope after long-term culture (cells were passaged every 3-5 days and kept culture for 21 days). A significantly higher proportion of EGFP-containing cells (~2.5%) were detected in the examined group when an HDR template with AAVS1 homology arms was provided, whereas only a few cells displayed EGFP signal in the control group (0.1%).

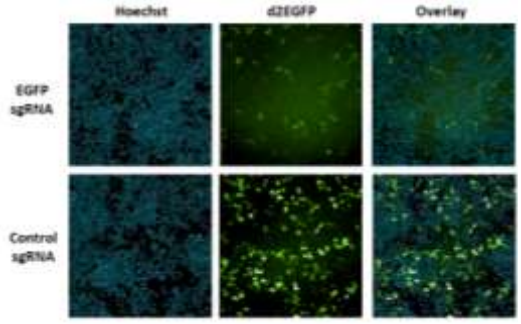


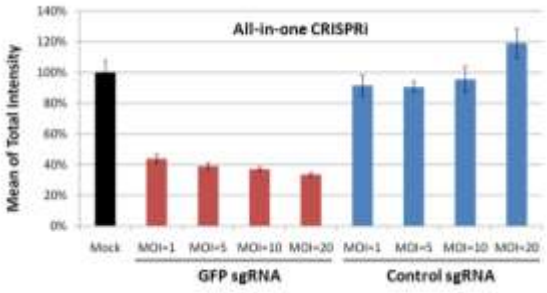
Part Ⅳ: CRISPR interference (CRISPRi)

Besides site-specific genome editing, CRISPR technique has been applied to modulate gene expression. In combination of sgRNA and dCas9-KRAB repressor protein, CRISPR/Cas system could specifically target to any gene of interest and thus repress its expression, which refers as CRISPR interference (CRISPRi). CRISPRi represses gene expression at transcriptional level by blocking transcription initiation (sgRNA targeting to promoter) or transcription elongation (sgRNA targeting to coding region).

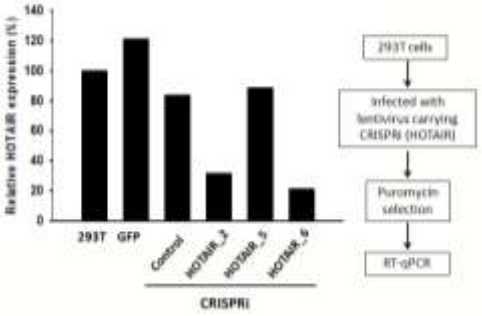


By targeting to the EGFP CDS, CRISPRi-mediated transcriptional repression could efficiently reduce the expression level of EGFP fluorescence after expressing EGFP sgRNA. Quantitative analysis showed that lentivirus-based CRISPRi could efficiently repress target gene (EGFP) expression even that the cells were transduced with low MOI of lentivirus (MOI =1).



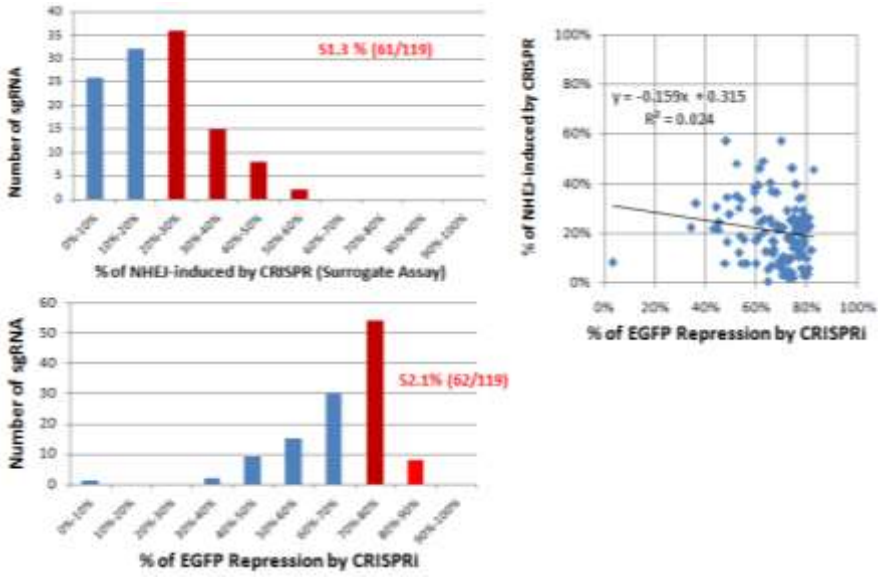


To further demonstrate that CRISPRi could efficiently repress endogenous gene (especially nuclear RNA, eg., lncRNA) expression, HEK293T cells were infected with lentivirus targeting the gene of HOTAIR lncRNA. After three days of puromycin selection, total RNA were harvested and the expression level of human HOTAIR lncRNA was analyzed by RT-qPCR. As expected, sgRNAs targeting to human HOTAIR gene (HOTAIR_2 and HOTAIR_6) were able to efficiently repress HOTAIR gene expression, whereas control sgRNA or sgRNA targeting to mouse HOTAIR gene (HOTAIR_5) did not significantly change the level of human HOTAIR lncRNA. The result indicates that CRISPRi is a powerful tool to repress nuclear target that is not possible to achieve it by using shRNA approach.



Part Ⅴ: Comparison of activities of CRISPR and CRISPRi

To compare whether sgRNAs have the same capability to work on CRISPR and CRISPRi, we generated 119 sgRNAs targeting EGFP and examined their activities in relation to the CRISPR-mediated indels and the CRISPRi-mediated repression by using a dual-fluorescence reporter. As aforementioned, the appearance of mCherry signal from the dual-fluorescence reporter indicates that there is an indel(s) formation in the EGFP CDS targeted by EGFP sgRNA(s). As shown in figure below, nearly 51.3% (61/119) of tested sgRNAs showed high frequency of NHEJ activities and induced up to 20% indels in the population of the tested cells. Among them, 52.1% (62/119) of the sgRNAs displayed good CRISPRi activities, which reduced the EGFP level by more than 70%. However, not all EGFP sgRNAs with good CRISPRi activities (EGFP repression) also showed high frequency of NHEJ activities (indel formation), indicating that the sgRNA(s) binding capability is not always consistent with its cleaving activity.



Comparative analysis of the activities of CRISPR/CRISPRi and the distance of sgRNA-binding site to transcription start site (TSS), the results showed that EGFP sgRNAs with higher NHEJ activities (higher frequency of indel formation) were primarily located within 200-bp from the TSS (left upper panel). On the other hand, there is no significant correlation between CRISPRi knockdown efficiency and the distance of EGFP sgRNA to TSS, of which sgRNAs with high CRISPRi activities target throughout the whole coding region of EGFP (left bottom panel). Moreover, sgRNAs with optimal GC content (50%-80%) might have the possibility to carry out higher NHEJ activities although not all these sgRNAs could efficiently induce indel formation in the tested cells. In

addition, sgRNAs with extreme low GC content (<50%) did not show good NHEJ activities and CRISPRi knockdown activity. These findings are consistent with previous reports published by others.