sgRNA level determines CRISPRi knockdown efficiency in K562 cells

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Abstract

***Objectives*:** To determine whether nuclease deactivated Cas9 (dCas9) or sgRNA expression level or both determines the knockdown efficiency of CRISPRi.

***Results*:** Cell clones expressing KRAB-dCas9 either under the control of the inducible Tet-on system or SFFV promotor were created by lentiviral transduction, and single clones were selected by fluorescence-activated cell sorting (FACS). Six genes with various expression levels were targeted using lentiviral sgRNA from two libraries, and we found that KRAB-dCas9 must reach a certain threshold for the knockdown. The

knockdown efficiency was neither affected by the target gene expression level nor does it correlate with the KRAB-dCas9 expression level, which remained relatively constant (CV=2.2%) across knockdown experiments. 74.72%, 72.28%, 39.08% knockdown of MMADHC, RPIA, ZNF148 genes were achieved in one cell clone, and the knockdown efficiency correlated well with the sgRNA expressing level, which is controlled by a different multiplicity of infection (MOI).

***Conclusions*:** Cell that expresses dCas9 above a certain threshold level can effectively knockdown target gene expression, and the knockdown efficiency correlated well with the sgRNA expression level.

Introduction

CRISPRi is gradually replacing the siRNA technique for mechanistic investigations in various fields (Evers et al. 2016). However, there is a lack of mechanistic study on the efficiency of the CRISPRi system. How the two major players of the system, the dCas9 fusion protein, and the guide RNA affect the knockdown efficiency has not been systematically studied.

Three protein domains were commonly used for repressing target gene transcription: KRAB, MeCP2, dCas9 and its synthetic derivatives (Gilbert et al. 2014; Yeo et al. 2018). KRAB recruits KAP1, which serves as a scaffold for various heterochromatin-inducing factors (Peng et al. 2009); MeCP2 binds to the methylated DNA and interacts with histone deacetylase and the corepressor SIN3A (Hansen et al. 2010). Combination of dCas9 with KRAB and MeCP2 has been shown to improve the knockdown efficiency

(Gilbert et al. 2014; Yeo et al. 2018), and dCas9, KRAB-dCas9, dCas9-KRAB-MeCP2 are three commonly used combinations.

Effective knockdown of target gene expression started from the sgRNA being expressed from the transgene, formed a complex with KRAB-dCas9 protein, and roaming in the nucleus until the sgRNA hybrid to target DNA (Ma et al. 2016). Previously inducible KRAB-dCas9 has been reported (Gilbert et al. 2014), but how knockdown efficiency was affected by dCas9 fusion protein or sgRNA expression level has not been studied systematically. In this study, we found that dCas9 protein must reach a certain threshold for an efficient knockdown, and the knockdown efficiency correlated well with the sgRNA expressing level.

Materials and methods

Cell culture

HEK293T and K562 cells were obtained from the National Infrastructure of Cell Line Resource. HEK293T cells were cultured at 37℃ in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose supplemented with 10% (v/v) Fetal Bovine Serum (FBS). K562 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS. Cells were tested every 3 months for mycoplasma contamination and consistently tested negative.

Construction of plasmids

The pTRE3G promoter was PCR-amplified with the primers using the pTRE3G plasmid as a template. The PCR product was digested with EcoRI and ligated into EcoRI

digested pHR-SFFV-KRAB-dCas9 vector (Addgene, 60954), to generate a pHR-pTRE3G-KRAB-dCas9 vector. Two complementary oligonucleotides were annealed and inserted into the BstX1 and Blp1 sites of the linearized pCRISPRia-v2 vector (Addgene, 84832) using T4 DNA ligase. Oligonucleotides are listed in Supplementary Table 1. Primers are listed in Supplementary Table 2.

Transfection and transduction

For transfection, 2.1×10^7 HEK293T cells were seeded into T175 flasks 1d before transfection. Cells were transfected with pSPAX2, pMD2.G, Tet3G/dCas9/sgRNA plasmid (4:1:4, mole ratio; Gilbert et al. 2014) using PEI (1 mg/ml) with N/P ratio was 20 (Yang et al. 2016). Viral supernatants were collected at 48 h and 72 h after transfection. For lentiviral concentration, the cell culture supernatant was filtered using 0.45 um filters. 4 ml 20% sucrose solution was added to the bottom of the ultracentrifuge tube with 32 ml filtered cell culture supernatant, centrifuge for 2 h at 25,000 rpm (82,700 g), 4 ℃ (Kutner et al. 2009), and the supernatant was disinfected and discarded. 100 ul of pre-cooled PBS without Ca^{2+}/Mg^{2+} was added to resuspend the pellet. The titer of the lentiviral vector was determined by fluorescence titering assay. For transduction, 1×10^5 K562 cells were infected with a mixture of lentivirus and 10 ug/ml polybrene.

FACS and antibiotics selection.

To generate KRAB-dCas9-mCherry-expressing stable cell lines, cells were analyzed based on the expression of mCherry by BD FACS Aria II and sorted into 96-well plates. After cell clones expanded, positive cells expressing mCherry were confirmed by flow

cytometry using CytoFLEX S. For the selection of cells expressing pLVX-Tet3G, 100 ug/ml G418 was applied for 10 days. For cells expressing sgRNA, 5 ug/ml puromycin was applied 72 h after infection, for an additional 72 h.

Western blot analysis and ELISA

Total protein was extracted using RIPA lysis and extraction buffer with 1 mM PMSF. Total protein concentration was measured by BCA protein assay kit. For Western blot analysis, equal amounts of total protein were loaded in 12% SDS-PAGE and transferred onto a 0.22 um nitrocellulose membrane at 100 V, 150 min. The primary antibody rabbit anti-human Cas9 Polyclonal Antibody (Clontech) was diluted 1: 2500 and the secondary antibodies IRDye^{TM}-800CW goat anti-rabbit and goat anti-mouse were diluted 1:12,000. Specific protein was detected by the Odyssey ^{TM} CLx Imaging system (Li-COR Bioscience). α-Tubulin was used as an internal control. For ELISA, the dCas9 protein was quantified using the CRISPR/Cas9 assay ELISA kit (Epigentek).

RNA isolation and qPCR

Total RNA was extracted using the Universal RNA Extraction Kit, and 1 ug RNA was reverse-transcribed using the PrimeScript RT master mix. The reaction system included 10 ul of SYBR Premix Ex Taq II, 6.4 ul of ddH_2O, 0.8 ul each of forward and reverse primers at 10 uM and 2 ul of cDNA, with the following cycling conditions: 95 ℃ for 30 s, and 45 cycles of 95 ℃ for 5 s, annealing temperature for 30 s, melting: 95 ℃ for 10 s, 65 ℃ for 60 s, 97 ℃ for 1 s, cooling: 37 ℃ for 30 s (LightCycler® 96, Roche). For relative quantification, RNA expression was normalized to actin, and the PCR amplification efficiency of primers was estimated by the slope of a standard curve.

qPCR primers are listed in Supplementary Table 2.

Statistical analysis

Data were expressed as mean ± SD of three biological replicates and the Student's *t*-test was applied for comparison between groups. $p<0.05$ was considered significant, $p<0.01$ was considered very significant, and $p<0.001$ was considered highly significant.

Results

Weissman lab lentiviral plasmid expressing KRAB-dCas9 under SFFV promoter were obtained and packaged in HEK293T cells with packaging plasmids, for obtaining cell clones that constitutively expressing KRAB-dCas9. To establish cell clones that express KRAB-dCas9 in doxycycline-inducible fashion, we replaced the SFFV promoter with doxycycline-inducible pTRE3G promoter (Fig. 1a). The Tet-on trans-activator rtetR-VP16 (Tet3G plasmid) was introduced into K562 cells by lentiviral infection, and the cells were selected using antibiotic G418. The inducible KRAB-dCas9 lentiviral plasmid was then packaged and used for infection into the G418 selected cells. The clonal K562 cells that either constitutively or inducible express KRAB-dCas9 were then selected by FACS utilizing the mCherry marker, and single clones of K562 cells were expanded (Fig. 1b). Two inducible clones, K562-idCas9 #49 and K562-idCas9 #60, and two constitutive clones, the K562-dCas9 #2 and K562-dCas9 #3 were obtained and infected with six different sgRNAs from two libraries to determine CRISPRi knockdown efficiency by qPCR (Fig. 1c).

We confirmed the inducible expression of KRAB-dCas9 in K562-idCas9 #49 by western blot and ELISA (Fig. 2a-b) and found that KRAB-dCas9 protein mainly localized in the nucleus, meeting expectation. Further titration of doxycycline and the corresponding KRAB-dCas9 expression were assessed using mCherry median fluorescent intensity (MFI), the tag that co-expressed with KRAB-dCas9, and similar response pattern was observed between ELISA and mCherry signal, indicating that mCherry reflected the KRAB-dCas9 expression level. Both inducible clones have a dose-responsive increase in mCherry MFI upon doxycycline induction (Fig. 2c). However, K562-idCas9 #49 has a uniform KRAB-dCas9 expression upon induction (Fig. 2d), while for K562-idCas9 #60 the expression is heterogenous (Fig. 2e). Similarly, K562-dCas9 #2 has a relatively universal KRAB-dCas9 expression (Fig. 2f), while the K562-dCas9 #3 clone has populations of low and high expression level, indicating either a mixture of cell clones or one clone with heterogeneous expression level (Fig. 2g). The inducible KRAB-dCas9 expressing clones has a much lower expression level compared to the constitutive clone K562-dCas9 #2, suggesting that inducible expression may not be able to reach the expression level of constitutive expression (Fig. 3a).

We assessed the knockdown efficiency of the four KRAB-dCas9 expressing clones using six different sgRNAs from two CRISPRi libraries. sgRNA was packaged into lentivirus and used for infection, and the cells expressing sgRNA were selected using puromycin. The knockdown efficiency was assessed by qPCR. K562-dCas9 #2, the clone with the highest KRAB-dCas9 expression level (Fig. 3a), has the highest knockdown efficiency; with all other clones has rather low or no knockdown efficiency

(Fig. 3b-g), indicating that a certain threshold of KRAB-dCas9 is required for efficiently knockdown. Among the six sgRNAs tested, MMADHC, RPIA, ZNF148, LSM4, and LIMA1 genes have been effectively knocked down, yet for the NPEPPS gene, no knockdown was achieved. We analyzed whether knockdown efficiency is affected by the target gene expression level, and found no correlation (Fig. 3h).

Utilizing K562-dCas9 #2, we assessed the impact of sgRNA expression level on knockdown efficiency. We infected K562-dCas9 #2 with sgRNA lentivirus at different MOI and determined the gene transfer rate using the BFP MFI, which is co-expressed with the sgRNA. We found an increase in the gene transfer rate, correlated with MOI, fitting a polynomial curve (Fig. 4a). Based on this curve, the gene transfer rate was 34.1% at MOI 0.5, the usually used MOI for CRISRPi library, which is close to the previously reported gene transfer rate 28.6% at MOI 0.5 in K562 cells (Kustikova et al. 2003), confirming an effective lentiviral packaging and transduction. BFP MFI in positive sgRNA expression cells linearly correlated with MOI, indicating that the sgRNA level increased with increasing MOI (Fig. 4b). Correspondingly, a linear relationship was observed between the MOI and the knockdown efficiency, as expected (Fig. 4c). In our research, based on the linear relationship between the MOI and the knockdown efficiency, around 45% knockdown could be achieved by MOI 0.5 for the MMADHC gene (Fig. 4c). Moreover, we studied the relationship between the KRAB-dCas9 expression level and the knockdown efficiency and found that the KRAB-dCas9 level remained relatively constant across different MOI of sgRNA infection (CV=2.2%), which does not correlate with the knockdown efficiency (Fig. 4d).

Discussion

We discovered that KRAB-dCas9 expression must reach a certain threshold for effective target gene knockdown, suggesting that care shall be taken when choosing cells/cell clones for CRISPRi applications. The requirement of a high KRAB-dCas9 level mandated selection of the cells after lentiviral infection. However, it is known that single cloning affected the transcription profile of the dCas9-KRAB expressing cells, suggesting that poly-clonal cell populations are better in CRISPRi applications (Stojic et al. 2018). The universal high expression of KRAB-dCas9 is hard to achieve in a heterogeneous population of cells. To meet the requirement of both high KRAB-dCas9 expression and heterogeneous cell population, selection for cells with enough KRAB-dCas9 level without single cloning shall be carried out, and such selection can be achieved by flow cytometry. More than one selection may be required, as the lentiviral transgene promoter could be methylated and transgene expression shut down in the continuous culture of the cells. A more potent CRISPRi system could be applied, too, such as the dCas9-KRAB-MeCP2, or the dCas9-MCP-KRAB-MeCP2 system (Martella et al. 2019).

We observed that once KRAB-dCas9 expression is high enough, CRISPRi efficiency depended on the sgRNA expression level. This observation corroborated the previous findings in CRISPR system, that the nuclear concentration of the guide RNA is the limiting factor for efficient DNA targeting (Yuen et al. 2017; Ma et al. 2016), indicating that the dynamics of ribonucleoprotein formation and DNA targeting is probably similar

between CRISPR and CRISPRi procedure. To improve the knockdown efficiency, it is critical to increasing the expression level of sgRNA, and a change of promotor could achieve this goal. Alternatively, since sgRNA is very unstable when it was not bound with Cas9 protein (Ma et al. 2016), improvement of the sgRNA stability could potentially increase efficiency. The correlation between the sgRNA expression level and the knockdown efficiency of the CRISPRi system has indications for the CRISPRi library screening: inducible sgRNA will provide better control of the knockdown. It has been reported that inducible sgRNA offered better control of knockout in the CRISPR system (Aubrey et al. 2015), again pointing to the similarities between the two tools.

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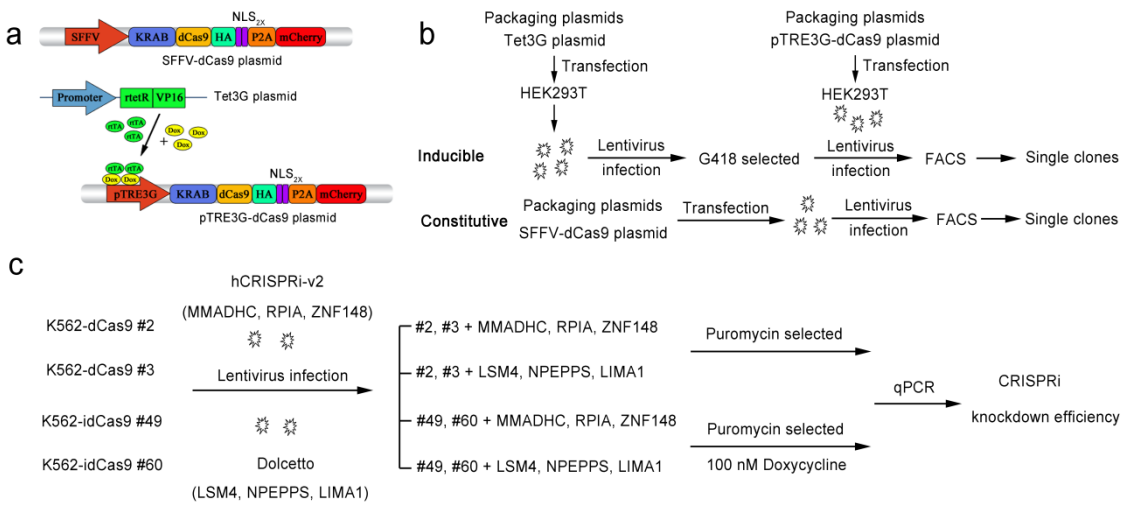

Fig. 1 Overview of the experimental procedure. **a** Vectors for inducible and constitutive KRAB-dCas9 expression. **b** Generate inducible and constitutive KRAB-dCas9 expressing cell clones using lentivirus. **c** Infect cell clones with sgRNA to determine CRISPRi knockdown efficiency.

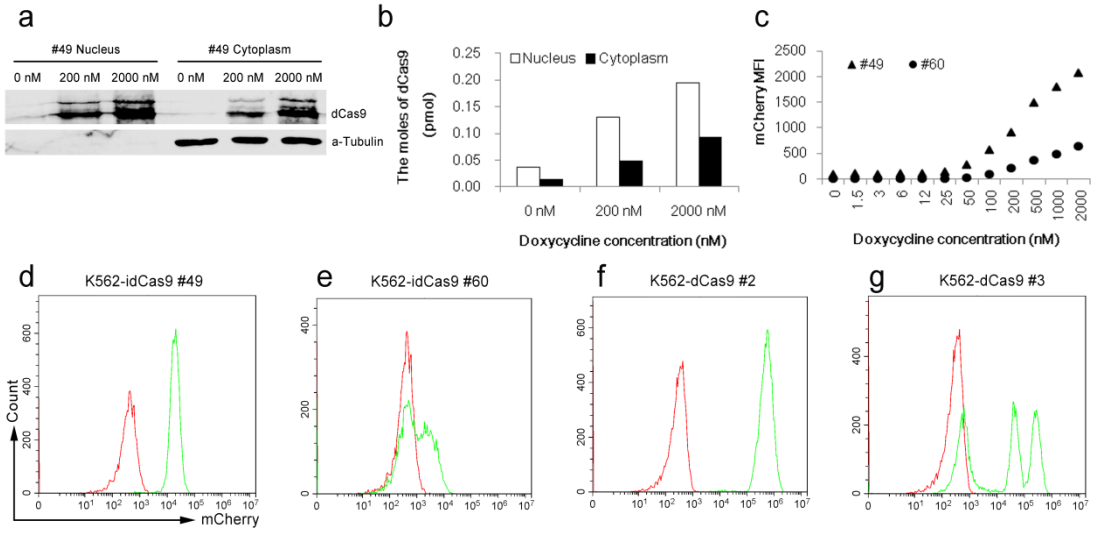

Fig. 2 The expression of KRAB-dCas9 in inducible and constitutive clonal cells. **a-b** Western blot and ELISA detection of nuclear and cytoplasmic KRAB-dCas9 in K562-idCas9 #49 induced by 200nM and 2000nM doxycycline. **c** mCherry MFI in

K562-idCas9 #49, K562-idCas9 #60, in response to various doses of doxycycline. Triangle indicated K562-idCas9 #49, round indicated K562-idCas9 #60. **d-e** The fluorescence intensity histogram of K562-idCas9 #49, K562-idCas9 #60 treated with 100 nM doxycycline, the red peak is the negative control K562 cells. **f-g** The fluorescence intensity histogram of K562-dCas9 #2 and K562-dCas9 #3, the red peak is the negative control of K562 cells.

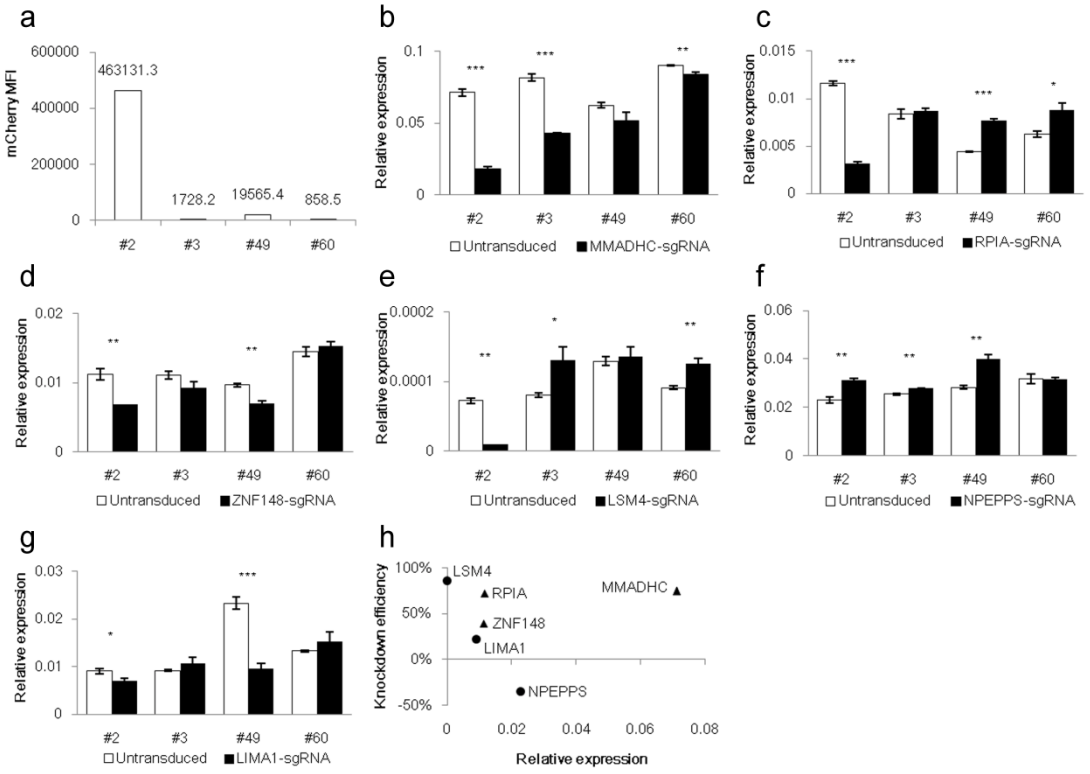

Fig. 3 Efficient CRISPRi knockdown depends on highly expressed KRAB-dCas9. **a** mCherry MFI of two inducible cell clones induced by 100 nM doxycycline and two constitutive cell clones. #49: K562-idCas9 #49; #60: K562-iCas9 #60; #2, K562-dCas9 #2; #3: K562-dCas9 #3. **b-g** CRISPRi knockdown of genes in the four-cell clones. Expression levels of target genes were determined by qPCR using actin as an internal

reference. Data are presented as the mean ± SD (n = 3). *p<0.05, **p<0.01, ***p<0.001. Untransduced is the target gene level in cell clone without sgRNA infection. **h** The relationship between target gene expression level and CRISPRi knockdown efficiency in K562-dCas9 #2. Triangle indicated hCRISPRi-v2 libraries. Round indicated Dolcetto.

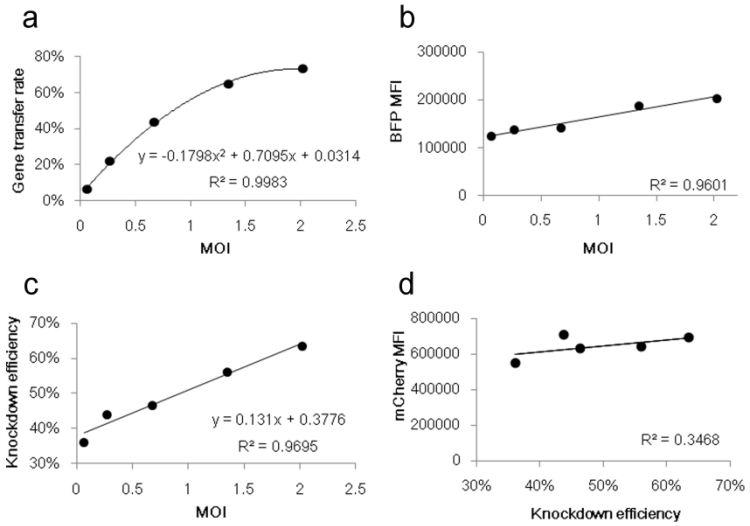

Fig. 4 CRISPRi knockdown efficiency correlated with sgRNA expression level. **a** Correlation between gene transfer rate and MOI of sgRNA infection of the MMADHC gene, determined by BFP MFI, in K562-dCas9 #2. **b** Correlation between BFP MFI in positive sgRNA expression cells and the MOI of sgRNA infection of the MMADHC gene in K562-dCas9 #2. **c** Correlation between knockdown efficiency and MOI of sgRNA infection of the MMADHC gene in K562-dCas9 #2. **d** Correlation between mCherry MFI and knockdown efficiency in K562-dCas9 #2.