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Transcriptional repression of endogenous genes in BmE cells using CRISPRi system

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ABSTRACT

Recent advancements in genetic engineering technology have led to the development of CRISPR interference (CRISPRi) as a precise tool for regulating gene expression. When CRISPR/dCas9 is fused with transcriptional repressors, the system can robustly silence endogenous gene expression. The CRISPR/Cas9 tool is a promising alternative in organisms (e.g., *Bombyx mori*) that do not respond to traditional gene suppression techniques, such as RNA interference (RNAi). However, transcriptional repressors remain poorly categorized in multiple cell types and species, leading to difficulties in optimizing performance and efficiency. Here, we tested CRISPRi usability and efficiency in *Bombyx mori* cells (BmE). We fused dCas9 to five transcriptional repressors including KRAB, Hairy, SID, SRDX, and ERD. All five constructs were efficient in BmE cells. In a proof-of-concept experiment, we showed that CRISPRi acting on *BmSoxE* (a gene involved in cell proliferation) could generate similar phenotypes as RNAi gene suppression. Moreover, CRISPRi has fewer off-target effects. Through co-transfection of BmE cells with sgRNAs, we also demonstrated that dCas9 could simultaneously repress the expression of multiple genes. Furthermore, we identified sgRNA distance from transcriptional start site (TSS) and the dCas9: sgRNA ratio as the two limiting factors of CRISPRi efficiency. Our results demonstrated that CRISPR/dCas9 is a viable and rapid alternative for functional investigations of the *B. mori* genome and perhaps other Lepidoptera insects.

1. Introduction

Since its initial application in *Caenorhabditis elegans* in 1998 (Fire et al., 1998), RNA interference (RNAi)-based gene silencing has been a useful method to determine gene function. The process is conserved and widespread in eukaryotes, involving mRNA degradation through the action of double-stranded RNA (dsRNA) (Fire, 2007). This method of gene silencing has been successfully used to control agricultural insect pests (Borovsky, 2005; Gordon and Waterhouse, 2007; Kim et al., 2015), most of which are Lepidoptera. Since its introduction as a model organism for this order (Quan et al., 2002), the silkworm, *Bombyx mori (B.mori)* has been frequently used in Lepidoptera RNAi studies, especially once its whole genome sequence became available (Xia et al., 2004). However, insects do not possess RNA-dependent RNA polymerase (RdRp), which mediates RNAi through amplifying exogenous dsRNA (Huvenne and Smagghe, 2010). Without RdRp, systemic RNAi is extremely difficult in most insects except some insects, such as, *Tribolium castaneum* (Fujita et al., 2006; Konopova and Jindra, 2008) and *Schistocerca gregaria (*Badisco et al., 2011; Boerjan et al., 2011*)*. In addition, a major shortcoming of RNAi is the presence of a strong off-target effect (Jackson et al., 2003; Lin et al., 2005; Ma et al., 2006), resulting in the silencing of genes that are only partially complementary with the original siRNA. The inherent weakness of RNAi, coupled with the lack of a key component in insects, suggest that RNAi has limited applicability in Lepidoptera.

The development of CRISPR/Cas9 gene-silencing system can potentially address the weaknesses of RNAi. The CRISPR/Cas9 method uses a protein-RNA complex to target and mediate gene modification (Hsu et al., 2013; Mali et al., 2013). Compared with traditional genome-editing tools like ZFN and TALEN, CRISPR/Cas9 is easier to implement and is therefore popular in many organisms (Bassett et al., 2013; Friedland et al., 2013; Hu et al., 2013; Jiang et al., 2013). This system is derived from a bacterial adaptive immune system, with numerous subfamilies. The type II CRISPR protein from *Streptococcus pyogenes*

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(*Sp*Cas9) is the most widely used (Jinek et al., 2012). A form of this protein lacking cleavage ability (dCas9) was developed to modulate gene expression in a method called CRISPR interference (CRISPRi) (Qi et al., 2013). CRISPRi mediated by dCas9 protein which binds on promoter region through limiting the transcriptional activity of RNA polymerase does not need any other transcription inhibitory factor components. However, silencing efficiency with a single dCas9 protein is very low, only about two-fold in human cells (Qi et al., 2013). To address this problem, researchers fused dCas9 with other transcription repressor domains such as the KRAB (Krüppel associated box) domain, the CS domain, dramatically improving CRISPRi efficiency by approximately 15-fold in human cells and 18-fold in yeast (Gilbert et al., 2013). In addition, dCas9 was also fused with the SRDX effector domain to regulate transcription in Arabidopsis (Piatek et al., 2015). These studies demonstrate that CRSIPRi is a powerful approach for performing loss-of-function experiments. However, we currently do not know whether dCas9 fused with repressors can be applied in Lepidoptera.

In this study, we performed dCas9-mediated transcriptional repression in BmE cells. We tested constructs of dCas9 fused with three other repressors, including the mSin3A interaction domain (SID) (Ayer et al., 1995; Eilers et al., 1999), the hairy domain (Fisher and Caudy, 1998; Paroush, 1994) and the ERF repressor domain (ERD) (Ohta et al., 2001; Sgouras et al., 1995). Our results should provide a foundation for dCas9-mediated transcriptional regulation in *B. mori*, while expanding the range of application for CRISPR/Cas9.

2. Materials and methods

2.1. dCas9 and sgRNA expression vector construction

The starting vectors were T-hr3/A4-dCas9 and T-U6-gRNA2, previously generated and stored in our laboratory. Codon-optimized transcriptional repressors were synthesized and inserted into pUC57-T-simple using genscript service. Transcriptional repressors were then fused to the C-terminal of T-hr3/A4-dCas9 via *SphI* and *Hin*dIII digestion and T4 ligation. All sgRNAs (Table 1) were synthesized as two complementary oligos by company service as two complemented oligos, then annealed and cloned into *Aarl*-digested T-U6-gRNA2. All plasmid vectors were sequenced before used.

2.2. Transfection of BmE cells

The BmE cell line was established from *B. mori* embryos kept in our laboratory. Cells were maintained at 27°Cin Grace medium supplemented with 10% FBS and penicillin-streptomycin. Before transfection, all plasmids were purified with a Plasmid Mini Kit (Qiagen), and BmE cells were seeded into 24-well plates with 70–90% confluent. A total of 1.0 μg plasmids were transfected into BmE cells using X-treme GENE HP DNA Transfection (Regeant) Roche according to the manufacturer's recommended protocols.

2.3. Western blotting analysis

Three days after transfection, proteins were extracted from BmE cells with NP-40 lysis buffer (Beyotime), following standard procedures. Cell lysate concentration was determined through a BCA assay, and 20 μg protein per sample was resolved on 8% SDS-PAGE. Samples were transferred to Polyvinylidene Fluoride (PVDF) membrane (GE Healthcare), then immunoblotted with anti-tubulin or anti-Cas9 antibody following standard procedures. The secondary antibody was used HRP-coupled sheep anti-mouse. Finally, Pierce ECL reagents were used to detect signals.

2.4. Immunofluorescence

dCas9-repressors were transfected into BmE cells with an 80%–90% density. Cells were washed with phosphate buffer saline (PBS) three times after transfection for 48 h. The primary antibodies were used: rabbit anti-dCas9 (1:500). The secondary antibody was used Alexa Fluor® 488 Goat Anti-rabbit IgG (H + L) (1:500, Beyotime, China). The nucleuses were stained by 4′,6-diamidino-2-phenylindole (DAPI). The cells were photographed using a confocal microscope (Olympus FV1000, Tokyo, Japan).

2.5. Quantitative RT-PCR analysis

3 days after transfection, total RNA was isolated using Total RNA kit II (Omega, USA) following the manufacturer's protocols. First-strand complementary DNA (cDNA) was synthesized from 1 μg RNA per sample, using 5 × PrimeScript RT Master Mix (Perfect Real time) (Takara, Japan). Quantitative real-time PCR (qRT-PCR) examination was performed using SYBR Premix Ex *Taq*II (Takara, Japan) on a 7500 fast Real-Time PCR system. All the primers sequences used in this study were list in Table 2.

Table 1
Design of sgRNA sequences in the study.

Targeted gene	sgRNA sequence(5′ to 3′)	Distance from TSS (from 3′ end of PAM)	Strand
BGIBMGA004612	ACGAATTTACACACCGCGCCCGG	−2	Non-template
	TCATAGATGTCGCTTGTTAGAGG	−36	Non-template
	AAAATGTTGATGCTCTCTGACGG	−92	Non-template
BGIBMGA002381	ATGTACTTCATGTACAAAAACGG	−27	Non-template
	TCACTAGCTCAGACGTCACTAGG	−83	Template
	TGTTCTGATTGGATAATTGACGG	−98	Non-template
BGIBMGA007469	GACTGTTGTAGGTGCTACGCAGG	−12	Non-template
	GATTTTGTGAACCCCGCGACAGG	−39	Non-template
	GCGGATATGTTGCTGACAGATGG	−62	Template
BGIBMGA006751	GTGAATTAAGAGTCAGTGTGAGG	−67	Template
	ACATTTGAAATAGTTTTACGTGG	−87	Non-template
	ACAAAATATATCTGATTGAAAGG	−144	Non-template
BGIBMGA003829	GCCCAGTGGATCGGCGCAAAAGG	−1	Template
	GTGGTACTGTATCGTTATGTTGG	−42	Non-template
	GGGAAATTAAAATGTTATATCGG	−78	Non-template
BGIBMGA011668	GTACTTACGCGCGGAGCATAAGG	−1	Non-template
	GCGCGCGCTCCCGACTGATATGG	−65	Template
	GCAGAAGAGCCATATCAGTCGGG	−51	Non-template

All the sgRNA sequences are displayed in **5′** to **3′** direction. The letters with underline represent PAM sequence.

Table 2
qRT-PCR primers used in this study.

Targeted gene	Forward primer(5′ to 3′)	Reverse primer(5′ to 3′)
SW22934	TTCGTACTGGCTCTTCTCGT	CAAAGTTGATAGCAATTCCCT
BGIBMGA004612	AGAACGAGGGCACTCTTACG	GATGTCGGCACCTGCTTGAA
BGIBMGA002381	CGATGAACCCCAACAACACAAT	TAGGTTTGCCTCCATCACTG
BGIBMGA007469	TTTCGTTATGCTGAAGGGTCG	TTGTGTGTGGAGGGACAGATA
BGIBMGA006751	GCATAGAGATTTGGTCCGTG	TGACTTAGCAGAAATGTCGTAG
BGIBMGA003829	ACTTCCTGGCTGGCGGTATCTC	TACCCTTGTAACGCTGGTCGGC
BGIBMGA011668	AACGCCGAACTCAGCAAATC	CGGGTGCTGTTTTTTATGTTG

2.6. Cell proliferation assay

BmE cells were seeded into 96-well plates and cultured in a final of 100 µl Grace medium. After transfection, cell proliferation was assayed at the following time points: days 1, 3, 5, and 7. Four hours before detection, cells were labeled with 10 µl of WST-8 solution (Cell Counting Kit-8; Dojindo). The absorbance was measured at 450 nm according to the manufacturer's protocol.

2.7. Flow cytometry detection

Cell-cycle distribution after dCas9-mediated transcriptional repression was analyzed with flow cytometry in FlowJo6.0 software. Before detection, Cells were stained with a Cell Cycle and Apoptosis Analysis Kit (Beyotime Biotechnology, China). After washing twice with PBS, cells were fixed with 70% ethanol at 4 °C. Subsequently, fixed cells were washed with PBS again and stained by propidium iodide (PI) with appropriate RNaseA. Finally, cells were checked at 488 nm on a flow cytometer (BD Biosciences, San Jose, CA, USA).

3. Results

3.1. Establishment of CRISPRi system in BmE cells

In previous study, we have constructed a CRISPR/Cas9 vector system that used hr3/A4 promoter to drive dCas9 containing a C-terminal nuclear localization sequence (NLS) (Fig. 1A). Here, we fused dCas9 with transcriptional repressors KRAB, Hairy, SID, ERD, and SRDX to test their ability in *B. mori*. We designed sgRNAs to target the upstream regions of target gene TSS according to the previous studies (Gilbert et al., 2014; Konermann et al., 2015). Three sgRNAs with increasing distance from the TSS (sgRNA1, sgRNA2, and sgRNA3) were designed per target gene, and they were cloned into the T-U6-gRNA2 vector. The expression of dCas9-repressors in BmE cells were determined with western blots (Fig. 1B). The results showed that all selected dCas9 repressor fusion proteins were translated efficiently in BmE cells. Furthermore, immunofluorescence analysis revealed that all the dCas9-fused proteins were located in the nucleus (Fig. 1C).

3.2. dCas9-repressor fusion can mediate transcriptional repression

To test whether CRISPRi system can suppress gene expression in BmE cells, we co-transfected five dCas9-repressor fusion proteins with sgRNAs into BmE cells respectively. As expected, all five dCas9-repressor fusion proteins successfully mediated the transcriptional repression of the five target genes: *BGIBMGA0004612* (4612), *BGIBMGA0002381* (2381), *BGIBMGA0007469* (7469), *BGIBMGA0006751* (6751), and *BGIBMGA0003829* (3829) (Fig. 2). In five cases, the dCas9-repressors can repress the expression of target genes. Statistical analysis showed that there were some significant differences among five dCas9-repressors in repressing each gene. And we found 3 repressors fusions: dCas9-SID, dCas9-SRDX and dCas9-ERD show slightly higher efficiency of gene repression. In addition, it is important to mention that not all of sgRNAs are targeting the template strand (T strand). sgRNA for gene 4612 all targeted non-template DNA strands (NT strand), whereas in gene 2381, only sgRNA1 and sgRNA3 targeted the NT strand, sgRNA2 targeted the T strand. For gene 7469, sgRNA1 and sgRNA2 targeted the NT strand while sgRNA3 targeted the T strand. We observed that both sgRNAs targeting NT strand and T strand showed equally high efficiency in repression. However, the results also showed that the gene repressions were different among sgRNAs for each gene. For gene 4612, sgRNA1 is not working with the dCas9-KRAB fusion protein. In other case, such as gene 2381, sgRNA1 was the most effective one among all the three sgRNAs. For gene 7469, all three sgRNAs exhibited high-efficiency silencing together with the five repressor fusion proteins. This suggested that the position of sgRNAs may also have effect on the repression efficiency.

To test whether dCas9 fused with repressors can mediate the repression of multiple genes at the same time in BmE cells, we chose the three most efficient fusion proteins, dCas9-SID, dCas9-SRDX or dCas9-ERD to target gene 7469, 3829, and 2381. One best sgRNA was used for each gene, sgRNA3 for gene 7469, sgRNA2 for 3829, and sgRNA1 for 2381. Our results demonstrated that the three repressor-fusion proteins can simultaneously and efficiently silence multiple genes in BmE cells (Fig. 3). And we found that dCas9-ERD exhibit the highest efficiency in two of three tested genes (Supplementary Fig. S1), so we decided to use the dCas9-ERD for subsequent experiments.

3.3. The gene repression mediated by CRISPRi is specific in BmE cells

The CRISPR/Cas9 system was reported to have activity with imperfectly matched sgRNA sequence to induce an off-target effect. Although dCas9 protein lacks the ability to cut the DNA sequence, we also detected the specificity of CRISPRi system in this study. Here, we predicted potential off-target genes of the most suitable sgRNA for genes 4612, 2381, 7469, 6751, and 3829 on the website https://crispr.cos.uni-heidelberg.de/. We found that sgRNA2 of 4612 targets *BGIBMGA013448*; sgRNA1 of 2381, *BGIBMGA005064*; sgRNA3 of 7469, *BGIBMGA002066*; sgRNA2 of 6751, *BGIBMGA003074*; and sgRNA2 of 3829, *BGIBMGA008066*. Then, we co-transfected BmE cells with dCas9-ERD and sgRNAs of the five target genes, we found no change in expression for the five corresponding off-target genes (Fig. 4). Therefore, dCas9-ERD was highly specific in target gene silencing.

3.4. CRISPRi suppresses BmE cell proliferation through repressing BmSoxE

To expand the application of CRISPRi system, we designed three sgRNAs to target upstream TSS of *BmSoxE* (*BGIBMGA011668*), a gene that had been silenced successfully using RNAi (Wei et al., 2014). We co-transfected dCas9-ERD and sgRNAs into BmE cells and demonstrated through qRT-PCR that all three sgRNAs could repress *BmSoxE* transcription, although sgRNA1 and sgRNA2 were more efficient than sgRNA3 (Fig. 5A). To investigate whether BmSoxE knockdown can mediate BmE cell proliferation, we further co-transfected the cells with dCas9-ERD, sgRNA1, and sgRNA2. At 7 d post-transfection, we observed a clear decrease in CRISPRi BmE cell count compared with control (Fig. 5B). Furthermore, to investigate the effect on BmE cells proliferation after transfecting dCas9-ERD and sgRNAs, BmE cells were

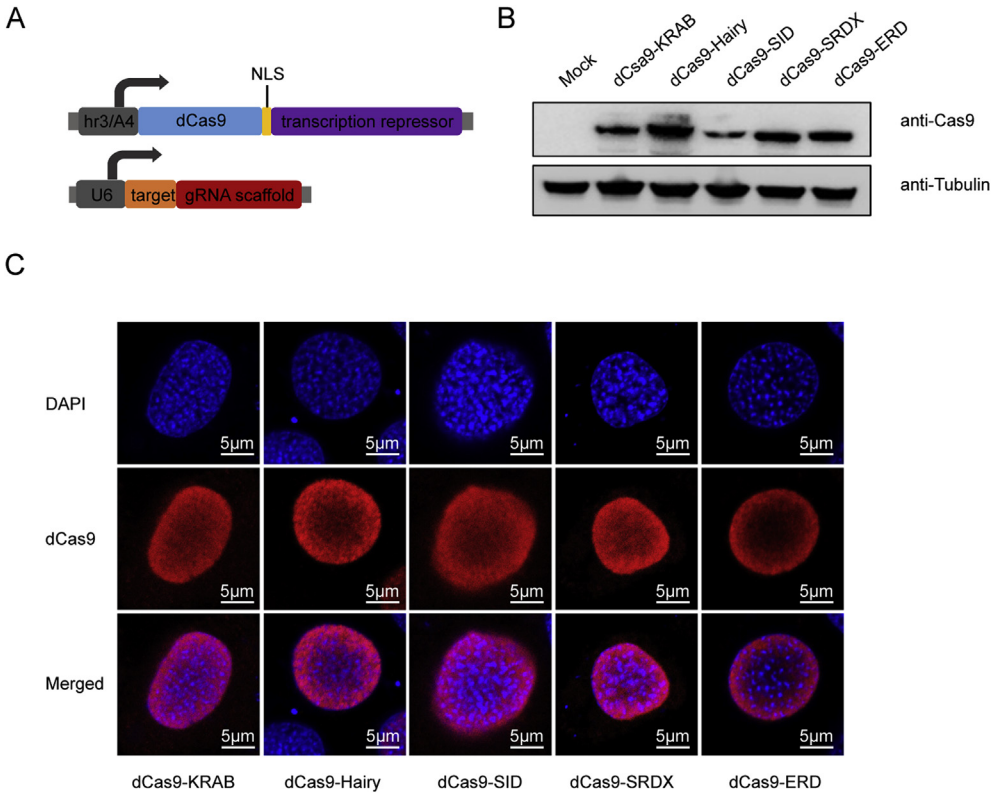

Fig. 1. Programmable engineered gene repression by dCas9.
A. Schematic of the constructs of CRISPRi system. Gray boxes indicate the hr3/A4 promoter and U6 promoter, respectively. Blue boxes indicate dCas9 which is from the type II CRISPR protein Streptococcus pyogenes and codon-optimized for silkworm. Golden boxes indicate NLS, nuclear localization signal, which is between the C terminal of dCas9 and transcriptional repressor (purple box). Orange box indicates the sequence of sgRNA target site. Dark red indicates the gRNA scaffold.
B. Western blotting analysis of dCas9 fused to repressors in BmE cells. Five repressive domains were fused to dCas9, including KRAB, Hairy, SID, SRDX, and ERD. Tubulin was used as control.
C. Immunofluorescence analysis of dCas9 protein in BmE cells. BmE cells were treated with anti-dCas9 (red). Nuclei were stained with DAPI (blue). The samples were observed by confocal microscopy. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

collected at different points, including 1st, 3rd, 5th, and 7th days after transfected, and the absorbance was detected at 450 nm. The results showed that cell proliferation of *BmSoxE* CRISPRi began to become slower compared to the control cells on the 3rd day, and reached a significant decrease on the 7th day (Fig. 5C).

As *BmSoxE* gene is related to cell cycle progression, we further performed a set of flow cytometry analyses to detect cells status after transfected dCas9-ERD with sgRNAs into BmE cells. The results

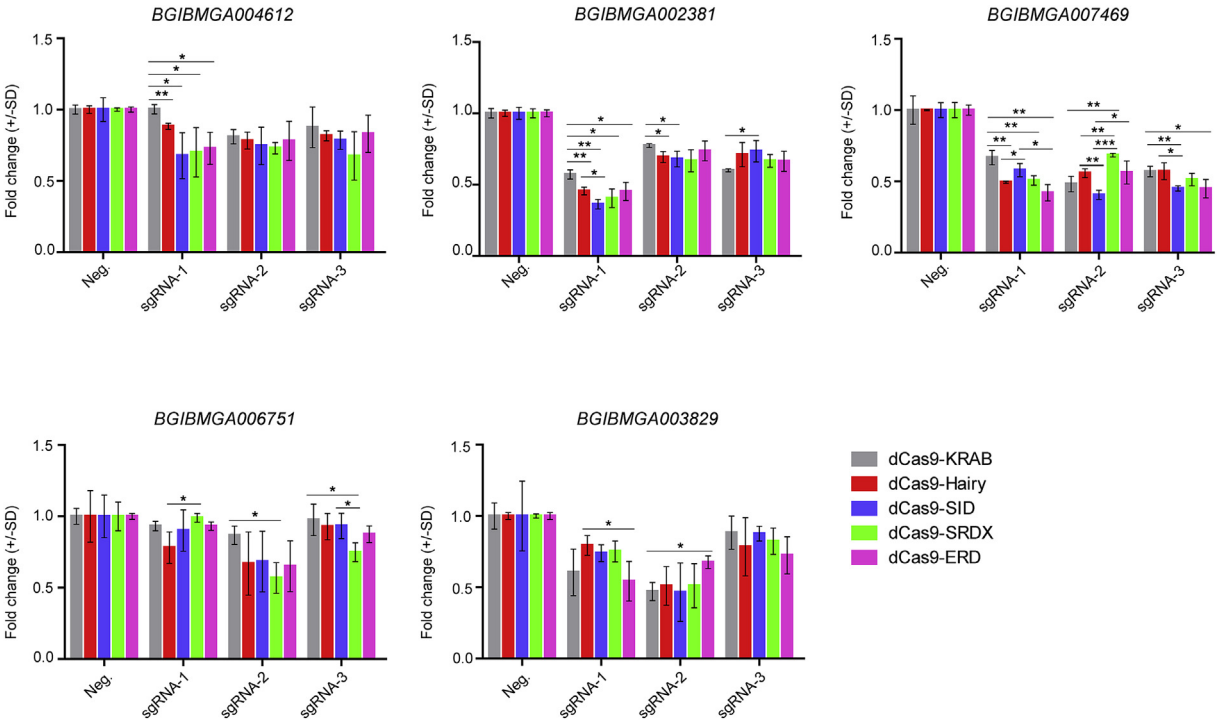

Fig. 2. Repression of endogenous genes by dCas9 fused to repressors in BmE cells. Three sgRNAs were designed for each gene and named sgRNA1, sgRNA2, and sgRNA3 according to the distance from TSS (transcription start site). BmE cells infected with dCas9 fused repressor and a sgRNA targeting EGFP were used as negative control. Cells were grown for 3 days and then analyzed for RNA expression by qRT-PCR. The data indicate the mean ± SD (n = 3). Statistical significance was assessed using Student's t-test. *P < 0.05; **P < 0.01; ***P < 0.001.

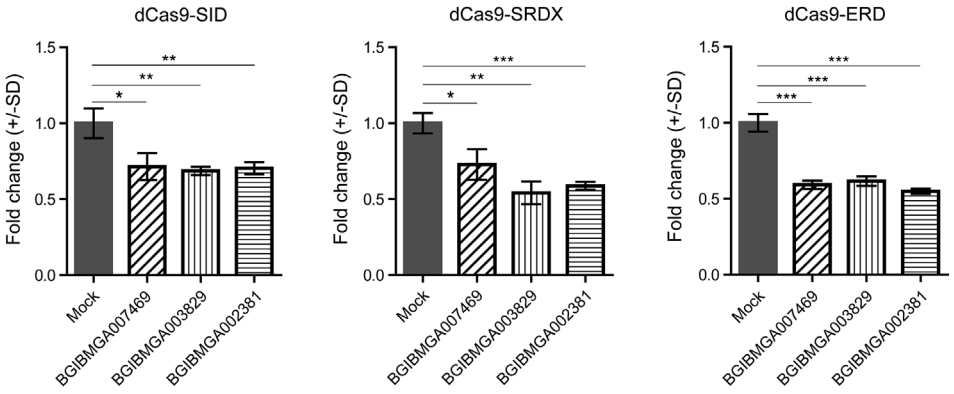

Fig. 3. Multiple gene repression by CRISPRi system. dCas9-SID, dCas9-SRDX or dCas9-ERD was combined with sgRNAs (one sgRNA for each gene which was used in Fig. 2) to target *BGIBMGA007469*, *BGIBMGA003829* and *BGIBMGA007469*. BmE Cells were grown for 3 days after transfection and were harvested for RNA expression analysis by qRT-PCR. Mock is BmE cells transfected with nothing. The data indicate the mean ± SD (n = 3). Statistical significance was assessed using Student's t-test. *P < 0.05; **P < 0.01.

revealed that on day 3 post-transfection, approximately 9% of transfected cells experienced cell cycle progression was arrested at the G1 phase (Fig. 5D). The *BmSoxE* CRISPRi cell cycle progression arrested at G1 phase was continuous with approximately 22% increase on the 7th day. The results of fluorescence-activated cell sorting (FACS) analyses confirmed that CRISPRi silencing of *BmSoxE* arrests BmE cells at G1 and negatively affects proliferation.

Furthermore, to compare the knockdown efficiencies between RNAi and CRISPRi in BmE cells, we performed siRNA silencing experiment to target the gene *BmSoxE* (*BGIBMGA011668*). For this gene, three siRNAs were designed. The result showed the knockdown by siRNA was about 20% whereas the knockdown by CRISPRi can reach about 50% (Supplementary Fig. S2). Taken together, the results demonstrated CRISPRi is a viable alternative to RNAi for mediating loss-of-function experiments in BmE cells.

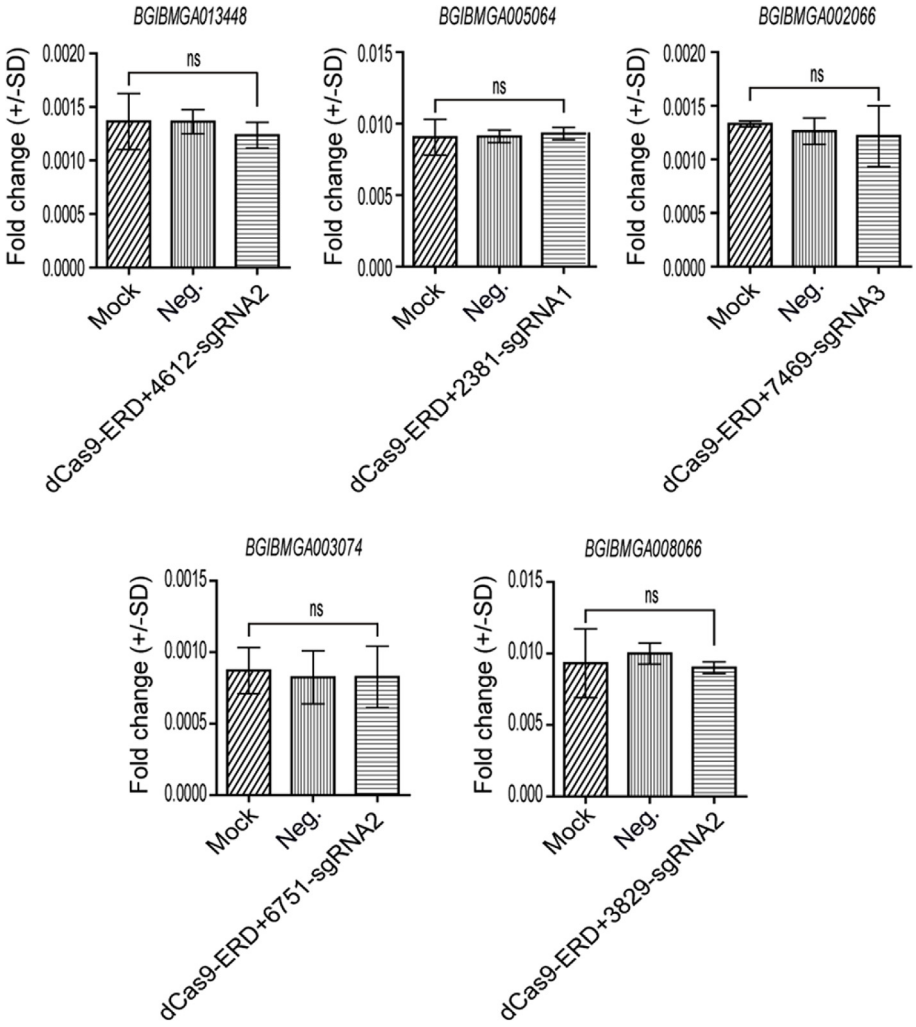

Fig. 4. CRISPRi is highly specific in BmE cells. BmE cells transfected with nothing were used as a Mock. BmE cells infected with dCas9-ERD and a sgRNA targeting EGFP were used as negative control. 3 days after transfection, the potential off-target genes repression levels were determined by qRT-PCR, and the data represent the mean ± SD (n = 3). Statistical significant differences were determined by Student's t-test, ns, no significant, compared with control.

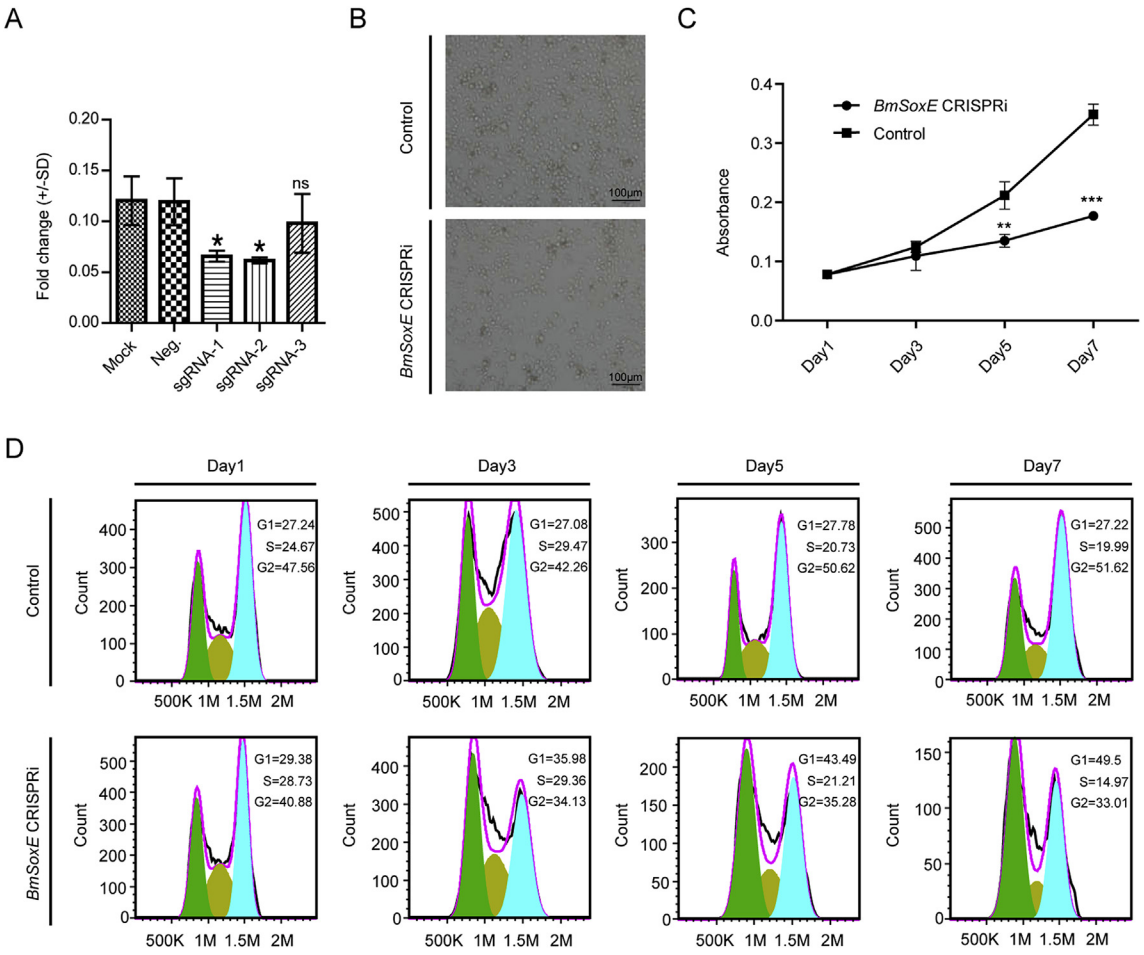

Fig. 5. Effects of *BmSoxE* CRISPRi on cell proliferation and cell cycle progression in BmE cells.

A. qRT-PCR detection of repression of *BmSoxE* gene by dCas9-ERD in BmE cells. Mock is BmE cells transfected with nothing. Negative control is BmE cells infected with dCas9-ERD and a sgRNA targeting EGFP. The data indicate the mean ± SD (n = 3). Significant difference was tested by Student's t-test, *P < 0.05; ns, no significant, compared with negative control.

B. Observation of cell proliferation by microscope. The number of *BmSoxE* CRISPRi cells was obvious reduced compared with control on the 7th day. BmE cells transfected with dCas9-ERD and a sgRNA targeting EGFP was used as control. Scale bar = 100 μm.

C. Cell proliferation curves of BmE cells after *BmSoxE* CRISPRi by absorbance detection at the pointed time compared with the control above. Data indicate the mean ± SD (n = 3 independent experiments). Significant difference was tested by Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001, P-value < 0.05 were considered as statistically significant.

D. Cell cycle progression analysis of BmE cells after *BmSoxE* CRISPRi by flow cytometry at the pointed time compared with the control above.

3.5. The repression efficiency of CRISPRi is related to the transfection ratio of dCas9: sgRNA

To obtain efficient transcriptional repression, we further explored whether the ratio of dCas9: sgRNA can affect the repression level. We co-transfected BmE cells with dCas9-ERD and sgRNA at different ratios to target gene 7469, gene 3829 and gene 2381(Fig. 6, Supplementary Fig. S3). As a result, we demonstrated that the repression was not dependent on the dose effect of dCas9 and the ratio of 1:4 is remarkable for a high repression. Therefore, sgRNA concentration has a stronger effect on the efficiency of transcriptional repression.

4. Discussion

In this study, we successfully developed a CRISPRi system in BmE cells by fusing dCas9 with different transcriptional repressors (KRAB, Hairy, SID, SRDX, and ERD). For the first time, we demonstrated that dCas9-fused proteins (specifically with ERD and Hairy) can knockdown and silence target-gene expression in BmE cells. Although the KRAB domain has been proved efficient in human cells, our results showed it was less so in BmE cells. For example, when targeted to gene 7469, ERD was a better option at sgRNA1 and sgRNA3, whereas SID was the most efficient at sgRNA2. Our results indicated the need to test more repressors to improve the ability of CRISPRi in future. Although the efficiency of these five repressors is no consistent at the same sgRNA site, we can also conclude that sgRNAs exert a bigger effect on repression efficiency than repressors. This indicates that we need to design more sgRNAs to screen the most highly efficient sgRNAs. Moreover, the concentration ratio of dCas9: sgRNA is also an important factor for effective repression, with 1:4 being the most effective configuration in this experiment.

We further confirmed that dCas9-repressor fusions can regulate multiple target genes at the same time in BmE cells, which suggests that CRISPRi could be suitable for high-throughput screening of genes in BmE cells. A notable competitor for this purpose is RNAi-mediated gene silencing with siRNAs or short hairpin RNAs (shRNAs), while siRNAs or shRNAs have high off-target effects (Ma et al., 2006). Notably, the activity of siRNAs and shRNAs are limited in silkworm cells. From those point of view, our data support that CRISPRi is a better tool for screens in silkworm cells. A comparison between CRISPRi and RNAi was performed to assess the reliability and efficiency for high-throughput screening in human cells (Housden and Perrimon, 2016; Morgens et al.,

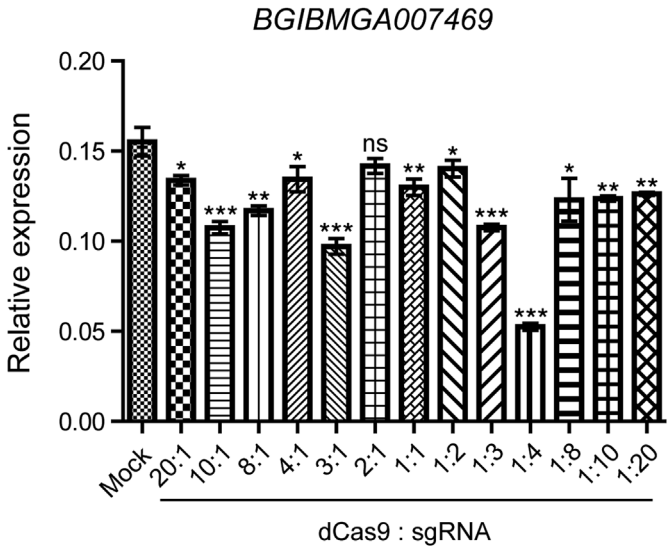

Fig. 6. Testing the effects of the ratio of dCas9 and sgRNA on repression efficiency.
BmE cells were transfected with indicated different ratios of dCas9-ERD and sgRNA1 targeted BGIBMGA007469. Mock is BmE cells transfected with nothing. Data indicate the mean ± SD (n = 3). Significant difference was tested by Student's t-test, *P < 0.05; **P < 0.01; ***P < 0.001; ns, no significant, compared with negative control.

2016). Candidate genes called from these two screens were not consistent, this may also indicate that RNAi-mediated screen is largely limited by its off-target effects. Although it was more specific than RNAi, CRISPRi system which used in this study was spCas9 has a limit of a suitable PAM site at the targeted gene. Together, these data suggested that CRISPRi and RNAi could be combined to identify more genes in large-scale genetic screening. In addition, we have used CRISPRi system to regulate the expression level of *BmSoxE* gene which can suppress the proliferation of BmE cells. This further demonstrated that CRISPRi system is valuable for genomic functional studies in BmE cells.

In conclusion, we have compared the efficacy of five transcriptional repressors, with ERD and Hairy used for the first time in CRISPRi. Next, we demonstrated that dCas9-repressor fusions are efficient tools for targeting either a single gene or multiple genes in BmE cells, advancing on previous CRISPR/Cas9 gene-editing studies in *B. mori* (Liu et al., 2014; Ma et al., 2014, 2017). Most importantly, our results significantly expanded CRISPR/Cas9 applicability and developed CRISPRi as a versatile tool to accelerate the study of gene function in silkworm and other insects.

Declaration of interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.05.007.

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