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A Powerful CRISPR/Cas9-Based Method for Targeted Transcriptional Activation

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Abstract: The targeted transcriptional activation of endogenous genes is an important tool for understanding physiological transcriptional networks, synthesizing genetic circuits, and inducing cellular phenotype changes. The CRISPR/Cas9 system has a great potential to achieve this purpose, however it has not yet succeeded to activate endogenous genes and induce cellular phenotype changes, efficiently. We herein show a powerful method for transcriptional activation by the CRISPR/Cas9-based system. Replacement of the methylated promoter with unmethylated one by the CRISPR/Cas9-based system was sufficient to activate the expression of the neural cell-specific gene *OLIG2* and the embryonic stem cell-specific gene *NANOG* in HEK293T cells. Moreover, CRISPR/Cas9-based *OLIG2* activation induced embryonic carcinoma cell line NTERA-2 to express neuronal marker β III-tubulin. Thus, these data provide a novel method to handle genetic circuits and phenotype changes.

Technologies for editing the epigenetic marks of targeted genes are required to understand complex transcriptional networks, accurately. Small molecules, such as DNA methylation inhibitors, alter the epigenetic state globally, but cannot target any specific loci. The CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeat/CRISPR-associated protein 9) system has been shown to target specific genomic locus and induce the site-directed DNA break, when combined with a single-guide RNA (sgRNA) that contains the complementary 20 nucleotides of the target sequence and the protospacer-adjacent motif (PAM), NGG^{[1]-[7]}. Recent studies have demonstrated that the nuclease-null Cas9 (dCas9), which was fused with transcription activation domains (e.g. VP16 or VP64), functioned as a transcriptional activator^{[8]-[18]}, suggesting that the CRISPR/Cas9-based system can be used to control the expression of specific genes. Since the transcriptional activity of the dCas9 activators was not high^[9],^[16], however, the efficacy of the cell-fate changes was still very low (~10%)^[17]. In addition, it was concerned that the dCas9 activators may trigger non-targeted transcriptional activation with unknown transcription regulators and their transcriptional activity may not be a physiological level. It was, therefore, required to establish new system that activates the target gene expression at physiological level and induces cell-fate changes.

In order to achieve this, we have focused on the microhomology-mediated end-joining (MMEJ)-dependent integration of donor DNA using CRISPR/Cas9^{[19], [20]}. MMEJ is a

DNA double-stranded break (DSB) repair mechanism that utilizes microhomologous sequences (5-25 bp) for error-prone end-joining^[21]. Using the MMEJ-combined CRISPR/Cas9 system, here we show a powerful method that activates the silenced gene by replacing its methylated promoter with unmethylated one. This system can be used for the analysis of transcriptional networks, cell-fates and genetic circuits in various types of cells.

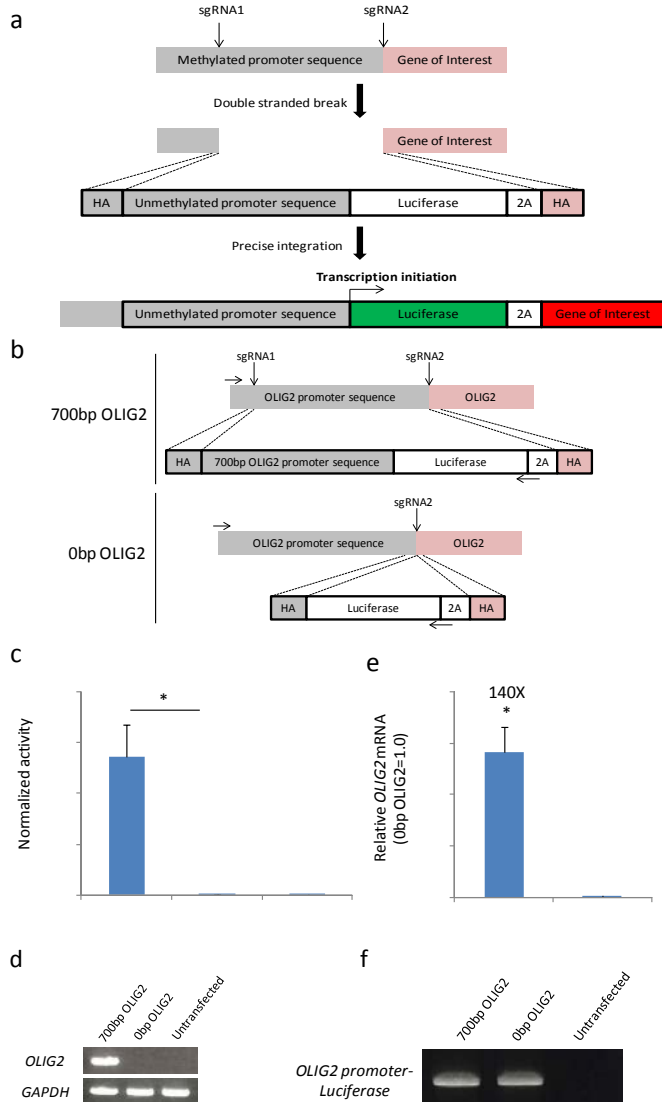
We designed a CRISPR/Cas9-based epigenome editing system (Fig. 1a). The methylated promoter in the targeted gene is cut off by two sgRNA-Cas9 complexes and replaced with the microhomology arms (HA)-harboring DNA fragment, which contains unmethylated promoter, luciferase and the foot-and-mouth disease virus 2A self-cleaving peptide (2A), by MMEJ-dependent integration, thereby we can monitor the transcriptional activity by measuring Luciferase activity. To examine our hypothesis, we targeted the *OLIG2* gene (Fig. 1b), which encodes a basic helix-loop-helix type transcription factor regulating neuronal and oligodendrocyte differentiation^{[22], [23]}. Since 700 bp upstream sequence from human *OLIG2* transcription start site (TSS), which has been registered as its promoter by the Transcriptional Regulatory Element Database (TRED), is reported as essential region for gene expression^[24],^[25], we constructed two vectors, the 0-bp *OLIG2* vector, which is for the insertion of the Luciferase gene and 2A into the *OLIG2* TSS, and the 700-bp *OLIG2* vector, which is for replacing the methylated *OLIG2* promoter with the unmethylated 700bp *OLIG2* promoter, the Luciferase gene and 2A, and transfected them into HEK293T cells. Five days after transfection, we found strong Luciferase activity (approximately 270-fold) in the 700-bp *OLIG2* vector transfected cells but not in the control cells (Fig. 1c). We also found the induction of *OLIG2* expression in the 700-bp *OLIG2* vector transfected cells by the end-point reverse transcriptase-PCR (RT-PCR) (Fig. 1d) and quantitative PCR (qPCR, approximately 140-fold compared with 0-bp *OLIG2* cells, Fig. 1e). Using western blotting analysis, we confirmed the accumulated *OLIG2* protein in the 700-bp *OLIG2* vector transfected cells (Supplementary fig. 1). Furthermore, FACS analysis revealed that *OLIG2* was expressed in the edited HEK293T cells (18.2% in total) at a same level as in human glioblastoma-initiating cell E6 (GBME6) (Supplementary fig. 7a left panels). We corrected that the 700-bp and 0-bp *OLIG2* were precisely integrated into the targeted genomic locus (Fig. 1f). These results revealed that our CRISPR/Cas9-based epigenome editing system can activate the transcription of an endogenous gene.

To address whether *OLIG2* induction by the epigenome editing system is sufficient to induce the cell-fate change, we applied this system to human embryonic carcinoma (EC) cells (NTERA-2). Four days after transfection, We found strong *OLIG2* expression in the 700-bp *OLIG2* cells by RT-PCR (Fig.

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2c) and qPCR (approximately 120-fold compared with 0-bp *OLIG2* cells, Fig. 2d). FACS analysis revealed that *OLIG2* was expressed in the edited NTERA-2 cells (19.1% in total) at a same level as in GBME6 (Supplementary fig. 7b). We corrected that the 700-bp and 0-bp *OLIG2* were precisely integrated into the targeted genomic locus (Fig. 2e). We observed morphological changes with axonal shapes in the 700-bp *OLIG2*-transfected cells at 7 days after transfection (Fig. 2a, Supplementary fig. 2a). These cells were immunolabeled for an early neuronal marker β III-tubulin (Fig. 2a) and a late neuronal marker neurofilament 160 (NF160, Supplementary fig. 2a),



whereas neither 0-bp *OLIG2* transfected cells nor untransfected cells were positive for these markers.

Figure 1. Activation of endogenous *OLIG2* gene expression by the CRISPR/Cas9-based epigenome editing system. (a) Schematic design of the CRISPR/Cas9-based epigenome editing system. (b) Schematic model of the *OLIG2* (700-bp and 0-bp) epigenome editing systems. Arrows indicate primer sites for genomic PCR. (c) Induced luciferase activity in the 700-bp *OLIG2* cells at 5 days after transfection. Error bars indicate SD (n=3). * $p < 0.01$ (d) RT-

PCR analysis of *OLIG2* transcription. (e) qPCR analysis of *OLIG2* transcription. Error bars indicate SD (n=3). * $p < 0.01$ (f) PCR evaluation of *OLIG2* epigenome editing using primers shown in (b).

FACS analysis further revealed that the approximately 20% of 700-bp *OLIG2* cells were positive for β III-tubulin and 10% of them were NF160-positive, although none of the 0-bp *OLIG2* cells were positive for these markers (Fig. 2b, Supplementary fig. 2b). These results apparently indicated that the epigenome editing of *OLIG2* promoter was sufficient to induce NTERA2 cells to differentiate into neurons.

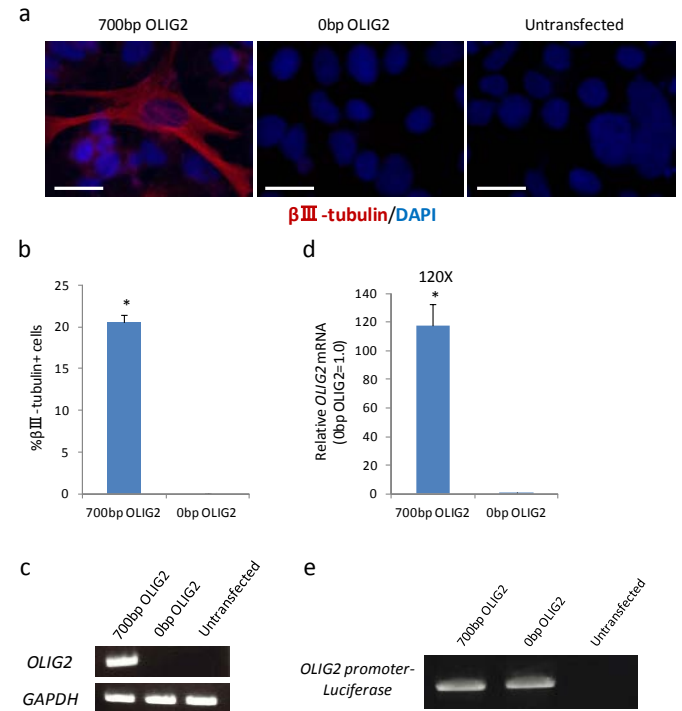


Figure 2. Induction of neuronal differentiation in NTERA2 cells using the CRISPR/Cas9-based epigenome editing system. (a) Immunostaining for the early neuronal marker β III-tubulin (Alexa568, Red) at 7 days after transfection. Nuclei were stained with DAPI (Blue). Bars= 50 μ m (b) FACS analysis of *OLIG2*- and β III-tubulin-positive cells at 7 days after transfection. Error bars indicate SD (n=3). * $p < 0.001$ (c) RT-PCR analysis of *OLIG2* transcription. (d) qPCR analysis of *OLIG2* transcription. Error bars indicate SD (n=3). * $p < 0.01$ (e) PCR evaluation of *OLIG2* epigenome editing. Integration events were assessed by genomic PCR using primers shown in Fig. 1b.

We then investigated the knock-in efficiency of the editing system. We transfected the 700-bp *OLIG2* vector with a GFP-expression vector (ratio 10:1) into NTERA-2 cells. Three days after transfection, we found that 52% of the cells were GFP+ in total (Supplementary fig. 3a, left panel). Among them, 39% of GFP+ cells were immunolabeled for *OLIG2* (Supplementary fig. 3a, middle panel). When NTERA-2 cells were transfected with DsRed-expression vector and GFP-expression vector (ratio 10:1), 96% of GFP+ cells, which were 52% of the transfected cells (Supplementary fig. 3a, left panel), were DsRed+ (Supplementary fig. 3a, right panel). BrdU-incorporation assay has revealed that both GFP+ and GFP- cells, which were

transfected with the 700-bp *OLIG2* vector, proliferated, similarly (Supplementary fig. 3b). Taken together, these data suggested that the knock-in frequency of the editing system was about 40%.

In order to evaluate whether the epigenome editing can activate other methylated promoters, we focused on *NANOG* that is an essential transcription factor required for the maintenance of pluripotency in embryonic stem (ES) cells and early embryos [26], [27]. Deb-Rinker et al. have shown the critical methylation sites at 300bp upstream of human *NANOG* TSS [28], therefore we targeted 700 bp upstream sequences from human *NANOG* TSS, which has been registered by the TRED, for the editing. We constructed two vectors, the 0-bp *NANOG* vector, which is for the insertion of the Luciferase gene and 2A into the *NANOG* TSS, and the 700-bp *NANOG* vector, which is for replacing the methylated *NANOG* promoter with the unmethylated 700bp *NANOG* promoter, the Luciferase gene and 2A, and transfected them into HEK293T cells (Fig. 3a). Four days after transfection, we found that *NANOG* was expressed in the edited HEK293T cells (18.6% in total) at a same level as in NTERA-2 cells (Supplementary fig. 7a right panels). We observed strong Luciferase activity (approximately 700-fold) in the 700-bp *NANOG* transfected cells but not in the control cells at 5 days after transfection (Fig. 3b). We verified the induction of *NANOG* expression in the 700-bp *NANOG* transfected cells by RT-PCR (Fig. 3c) and qPCR (approximately 280-fold compared with 0-bp *NANOG* cells, Fig. 3d). Western blotting analysis further verified the induction of *NANOG* protein in the 700-bp *NANOG* vector transfected cells (Supplementary fig. 1). A genomic PCR analysis indicated that the 700-bp and 0-bp *NANOG* were precisely integrated into the genomic locus (Fig. 3e). Together all, these data suggested that the epigenome editing can be used to activate silenced promoters.

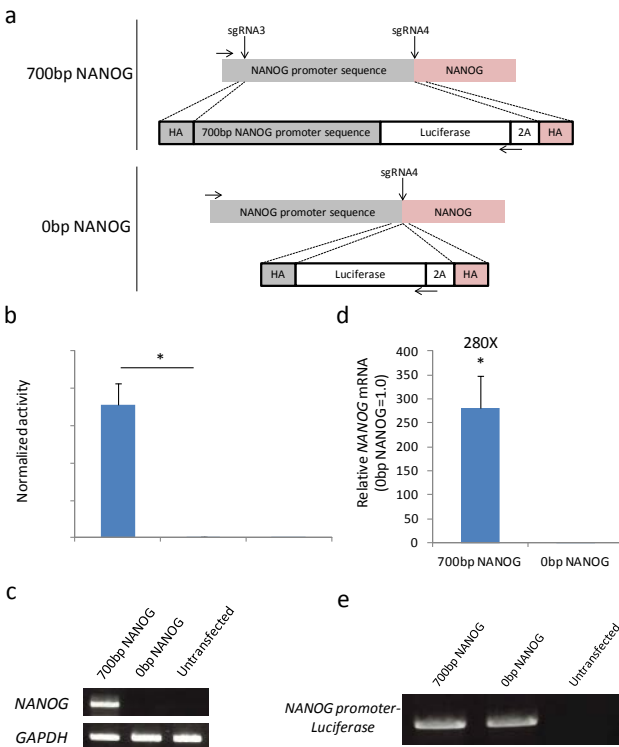


Figure 3. Activation of *NANOG* gene in HEK 293T cells by the CRISPR/Cas9-based epigenome editing system. (a) Schematic model of the 700-bp *NANOG* and 0-bp *NANOG* epigenome editing systems. Arrows indicate primer sites for genomic PCR. (b) Induced luciferase activity by the 700-bp *NANOG* editing. Error bars indicate SD ($n=3$). * $p<0.001$ (c) RT-PCR analysis of *NANOG* transcription. (d) qPCR analysis of *NANOG* transcription. Error bars indicate SD ($n=3$). * $p<0.001$ (e) PCR evaluation of *NANOG* epigenome editing using primers shown in (a).

To clarify whether our epigenome editing system induced off-target mutations, we selected two highest potential off-target sites of each gRNA, which were ranked by the CRISPR design tool (<http://crispr.mit.edu/>). We amplified the target sites by PCR (Supplementary fig. 4a) and evaluated their sequences. There was no mutation in the potential off-target sites (Supplementary fig. 4b).

Cas9-mediated genome editing has been used to various types of cells and model organisms [29]. In particular, the application of this system to human cells including pluripotent stem cells is useful for making disease models and finding therapeutic methods [30]-[33]. These indicate that the CRISPR/Cas9 system has great potential for broad applications.

Since the finding of dCas9, a number of transcriptional activation system based on the CRISPR/Cas9 system have been developed extensively. For instance, Nihongaki et al. and Polstein et al. have developed the CRISPR/Cas9-based photoactivatable transcription system, which consists of dCas9, light-sensitive CRY2, its binding partner CIB1, and p65 activation domain [34], [35]. This system induced a rapid transcriptional activation within a few hours after the blue light irradiation, however transcriptional activity was not high enough to induce cell fate conversion. Hilton et al. have combined CRISPR/Cas9 with p300 acetyltransferase [36]. This system could activate the expression of target genes but has not succeeded to induce cell differentiation in human pluripotent stem cells. On the other hand, our epigenome editing system has proved to activate *OLIG2* expression in NTERA2 cells and induce their neuronal differentiation, by only replacing the methylated promoter with unmethylated one. However, our method has a limitation that cannot activate various genes simultaneously, whereas Konermann et al. have demonstrated by using dCas9-VP64 system [8].

To construct vectors with unmethylated target promoter, we have extensively used *E. Coli* DH5 strain (*hsdR17(rk-,mk+)*, *recA1*, *relA1*, *supE44*, *thi-1*, *endA1*, *gyrA96*) [37] competent cells. Although most of competent strains contain three DNA methyltransferases, Dam (methylates adenine in GATC), Dcm (methylates internal cytosine in CCAGG and CCTGG) and EcoK1 (methylates adenine in AAC(N6)GTGC and GCAC(N6)GTT), none of these methyltransferases target CpG that mammalian DNA methyltransferases, Dnmt1, Dnmt3a and 3b, recognize [38]. In addition, our data showed that unmethylated *OLIG2* and *NANOG* promoters induced the expression of endogenous *OLIG2* and *NANOG* mRNA as well as luciferase gene, indicating that the methyltransferase activities in *E. coli* DH5 strain would not affect in our system.

As we have demonstrated, replacement of the methylated promoter with unmethylated one was sufficient to activate the target gene expression. However, this raised a question of how long the unmethylated status was kept in the cells. Both Dnmt3a and 3b were shown to induce de novo methylation at CpG sites, therefore methylation kinetics in the edited promoter seems to depend on their expression level. In the mouse ES cells that highly express both Dnmt3a and 3b^[39], it has been shown to take 11 days until a de-methylated locus by 5-Aza has been remethylated to a steady-state^[40]. The Human Protein Atlas data (<http://www.proteinatlas.org/>) indicated that the expression of Dnmt3a and 3b is relatively low in HEK293T, whereas NTERA2 strongly expresses both genes. Since we have detected *OLIG2* expression in NTERA2 at 7d after transfection (Fig. 2c and 2d), the edited promoter should keep the active state in the cells by at least 7d. In the case of HEK293T, we detected *OLIG2* expression in the cells at 21d after transfection (Supplementary fig. 5). Thus, our epigenome editing system activates target gene expression transiently, depending on the expression level of Dnmt3a and 3b. However, it may be possible to achieve a long-lasting active state of the specific promoter by coupling our epigenome editing system with a DNA demethylase, such as TET1 hydroxylase^[41].

The DNA demethylating drug 5-aza-2-deoxycytidine (5-Aza) is broadly used to erase DNA methylation in the genome wide^[42] and to activate genes that are silenced by their promoter methylation. We have used 5-Aza to induce *OLIG2* expression in HEK293T and NTERA2, however we could not detect the expression in these cells (Supplementary fig. 6), indicating that 5-Aza does not always activate any gene expression.

Altogether, these indicate that our epigenome editing system is suitable for understanding complex transcriptional networks at a physiological level, synthesizing genetic circuits, and engineering cell.

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Keywords: Synthetic Biology • Gene technology • DNA methylation • Gene expression • mRNA

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