Gene Editing

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

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Abstract: *Targeted transcriptional activation of endogenous genes is important for understanding physiological transcriptional networks, synthesizing genetic circuits, and inducing cellular phenotype changes. The CRISPR/Cas9 system has great potential to achieve this purpose, however, it has not yet been successfully used to efficiently activate endogenous genes and induce changes in cellular phenotype. A powerful method for transcriptional activation by using CRISPR/Cas9 was developed. Replacement of a methylated promoter with an unmethylated one by CRISPR/Cas9 was sufficient to activate the expression of the neural cell gene OLIG2 and the embryonic stem cell gene NANOG in HEK293T cells. Moreover, CRISPR/Cas9-based OLIG2 activation induced the embryonic carcinoma cell line NTERA-2 to express the neuronal marker βIII-tubulin.*

Methods for editing the epigenetic marks of targeted genes are required to understand complex transcriptional networks accurately. Small molecules, such as inhibitors of DNA methylation, alter the epigenetic state globally, but cannot target any specific loci. The clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system has been shown to target specific genomic loci and induce site-directed DNA breaks 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 a nuclease-null Cas9 (dCas9) fused to transcription activation domains (e.g., VP16 or VP64) functions as a targeted transcriptional activator,[8–18] thus suggesting that CRISPR/Cas9 can be used to control the expression of specific genes. Since the transcriptional activity of the dCas9 activators was not high,[8,16] however, the efficacy of the cell-fate change was low (ca. 10%).[17] In addition, it was a concern that the dCas9 activators might trigger non-targeted transcriptional activation with unknown transcription regulators, and that their transcriptional activity may not be at physiological levels. A new system is thus needed that activates the expression of target genes at physiological levels and efficiently induces cell-fate changes.

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In order to achieve this, we focused on the microhomology-mediated end-joining (MMEJ)-dependent integration of donor DNA by using CRISPR/Cas9.[19,20] MMEJ is a DNA double-strand break (DSB) repair mechanism that utilizes microhomologous sequences (5–25 bp) for error-prone end-joining.[21] Combining MMEJ with CRISPR/Cas9 enabled the development of powerful method by which a silenced gene is activated by replacing the methylated promoter with an unmethylated one. This system can be used for the analysis of transcriptional networks, cell-fate, and genetic circuits in various types of cells.

Our CRISPR/Cas9-based epigenome editing system is shown in Figure 1a. The methylated promoter in the targeted gene is cut out by two sgRNA-Cas9 complexes and replaced with the microhomology arm (HA)-harboring DNA fragment, which contains the unmethylated promoter, a luciferase reporter, and the foot-and-mouse disease virus 2A self-cleaving peptide (2A), by MMEJ-dependent integration. The transcriptional activity can then be monitored by measuring luciferase activity. To test our system, we targeted the *OLIG2* gene (Figure 1b), which encodes a basic helix-loop-helix transcription factor that regulates neuronal and oligodendrocyte differentiation.[22,23] Since the 700 bp sequence upstream of the human *OLIG2* transcription start site (TSS) has been registered as its promoter in the Transcriptional Regulatory Element Database (TRED) and is reported as an essential region for gene expression,[24,25] we targeted this region. Two vectors were constructed, the 0-bp *OLIG2* vector for insertion of just the luciferase gene and 2A into the *OLIG2* TSS as a control, and the 700-bp *OLIG2* vector for replacing the methylated *OLIG2* promoter with the unmethylated 700 bp *OLIG2* promoter, the luciferase gene and 2A. These vectors were transfected into HEK293T cells, and five days later, we found strong luciferase activity (approximately 270-fold over background) in the cells transfected with the 700-bp *OLIG2* vector but not in the control cells (Figure 1c). We also detected induction of *OLIG2* expression in the cells transfected with the 700-bp *OLIG2* vector transfected by end-point reverse transcriptase PCR (RT-PCR; Figure 1d) and quantitative PCR (qPCR, approximately 140-fold increase compared to cells transfected with 0-bp *OLIG2*, Figure 1e). Through western blotting, we confirmed accumulation of the OLIG2 protein in the cells transfected with the 700-bp *OLIG2* vector (Figure S1 in the Supporting Information). Furthermore, flow cytometry revealed that OLIG2 was expressed in the edited HEK293T cells (18.2% in total) at a same level as in human glioblastoma-initiating cells E6 (GBME6; Figure S2a left panels). We confirmed that the 700-bp and 0-bp *OLIG2* sequences were precisely integrated into the targeted genomic locus by PCR (Figure 1f). These results

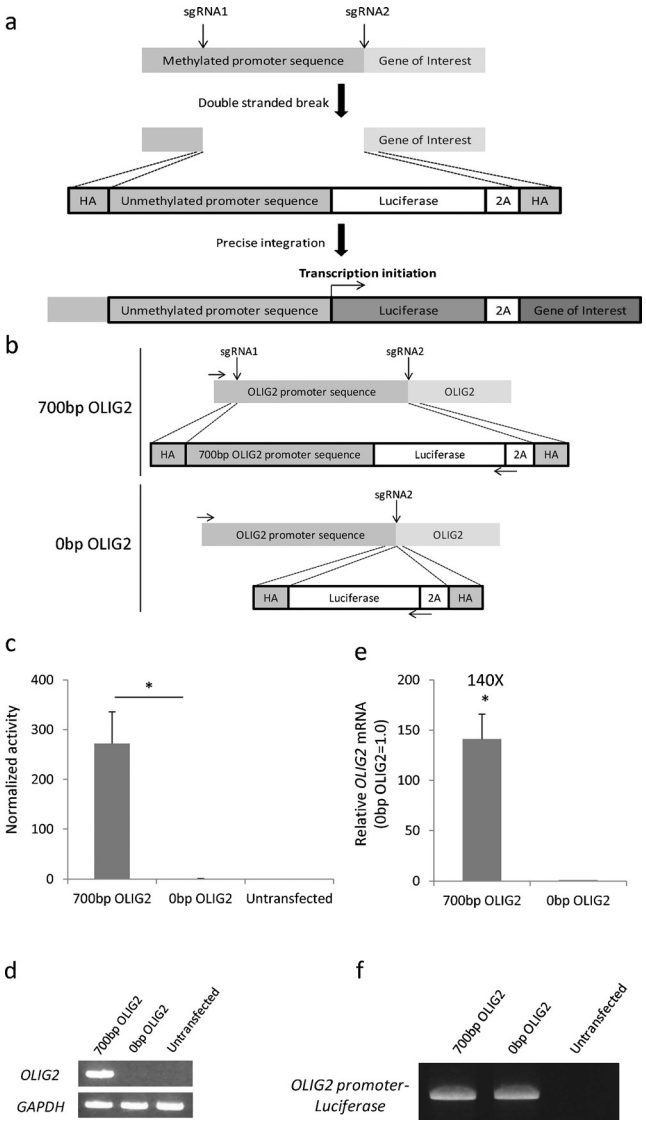

Figure 1. Activation of endogenous *OLIG2* gene expression by the CRISPR/Cas9-based epigenome editing. a) Scheme of the CRISPR/Cas9-based epigenome editing system. b) The *OLIG2* (700-bp and 0-bp) epigenome editing vectors. Arrows indicate primer sites for genomic PCR. c) Induced luciferase activity in the 700-bp *OLIG2* cells 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 the primers shown in (b).

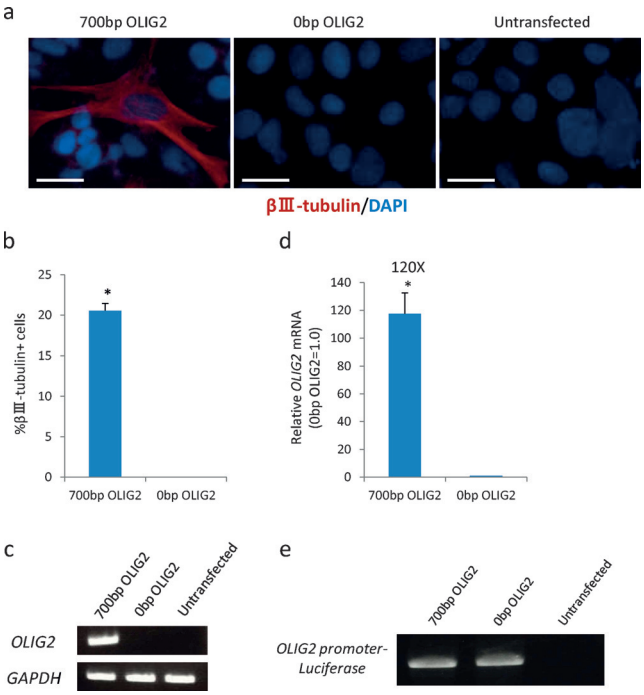

Figure 2. Induction of neuronal differentiation in NTERA-2 cells by using the CRISPR/Cas9-based epigenome editing system. a) Immunostaining for the early neuronal marker βIII-tubulin (AlexaFluor568, red) 7 days after transfection. Nuclei were stained with DAPI (blue). Scale bars: 50 μm. b) Flow cytometry analysis of OLIG2+ and βIII-tubulin+ cells 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 Figure 1b.

revealed that our CRISPR/Cas9-based epigenome editing system can be used to activate the transcription of an endogenous gene.

To determine whether *OLIG2* induction by the epigenome editing system is sufficient to induce a change in cell fate, we applied this system to the human embryonic carcinoma (EC) cell line NTERA-2. Four days after transfection, we measured strong *OLIG2* expression in the cells transfected with 700-bp *OLIG2* by RT-PCR (Figure 2c) and qPCR (approximately 120-fold increase compared with 0-bp *OLIG2* cells; Figure 2d). Flow cytometry revealed that OLIG2 was expressed in the edited NTERA-2 cells (19.1% in total) at the same level as in GBME6 cells (Figure S2b). We confirmed that the 700-bp and 0-bp *OLIG2* sequences were precisely integrated into the targeted genomic locus by PCR (Figure 2e). We observed morphological changes with axonal shapes in the 700-bp *OLIG2* cells 7 days after transfection (Figure 2a, Figure S3a). The cells were immunolabeled for the early neuronal marker βIII-tubulin (Figure 2a) and the late neuronal marker neurofilament 160 (NF160, Figure S3a), and the 700-bp *OLIG2* cells showed positive staining, whereas neither the 0-bp *OLIG2* cells nor untransfected cells were positive for these markers.

Flow cytometry further revealed that approximately 20% of 700-bp *OLIG2* cells were positive for βIII-tubulin expression and 10% of them were positive for NF160 expression, although none of the 0-bp *OLIG2* cells were positive for these markers (Figure 2b, Figure S3b). These results indicate that epigenome editing of the *OLIG2* promoter was sufficient to induce NTERA-2 cells to differentiate into neurons.

We then investigated the knock-in efficiency of the editing system. We transfected the 700-bp *OLIG2* vector along 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 positive (GFP+, Figure S4a, left panel). Of these, 39% showed positive immunolabeling for OLIG2 (Figure S4a, middle panel). When NTERA-2 cells were trans-

fected with a DsRed expression vector and a GFP expression vector (ratio 10:1), 96% of the GFP+ cells, which were 52% of the transfected cells (Figure S4a, left panel), were also positive for DsRed (Figure S4a, right panel). A BrdU-incorporation assay has revealed that GFP+ and GFP− cells that were transfected with the 700-bp *OLIG2* vector proliferated at similar rates (Figure S4b). Taken together, these data suggest that the knock-in frequency of the editing system is about 40%.

In order to evaluate whether this epigenome editing method can be used to activate other methylated promoters, we focused on NANOG, which 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 that the critical methylation sites are 300 bp upstream of the human *NANOG* TSS,[28] therefore, we targeted the 700 bp sequences upstream of the human *NANOG* TSS, which has been registered in TRED, for editing. We constructed two vectors, the 0-bp *NANOG* vector for insertion of just the luciferase gene and 2A into the *NANOG* TSS as a control, and the 700-bp *NANOG* vector for replacing the methylated *NANOG* promoter with the unmethylated 700 bp *NANOG* promoter, the luciferase gene, and 2A, and transfected them into HEK293T cells (Figure 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 (Figure S2a right panels). We observed strong luciferase activity (approximately 700-fold over background) in the 700-bp *NANOG* cells but not in the control cells 5 days after transfection (Figure 3b). We verified the induction of *NANOG* expression in the 700-bp *NANOG* cells by RT-PCR (Figure 3c) and qPCR (approximately 280-fold increase compared to the 0-bp *NANOG* cells, Figure 3d). Western blotting analysis further verified the induction of NANOG protein in the cells transfected with the 700-bp *NANOG* vector (Figure S1). A genomic PCR analysis indicated that the 700-bp and 0-bp *NANOG* sequences were precisely integrated into the genomic locus (Figure 3e). Taken together, these data suggest that this epigenome editing method can be used to activate silenced promoters.

To clarify whether our epigenome editing system induces off-target mutations, we selected the 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 (Figure S5a) and evaluated their sequences, and we found no mutations (Figure S5b).

Cas9-mediated genome editing has been used in 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] and indicates that the CRISPR/Cas9 system has great potential for broad applications.

Since the discovery of dCas9, a number of transcriptional activation systems based on the CRISPR/Cas9 system have been developed. For instance, Nihongaki et al. and Polstein et al. have developed a CRISPR/Cas9-based photoactivatable transcription system, which consists of dCas9, light-sensitive CRY2, its binding partner CIB1, and the p65 activation

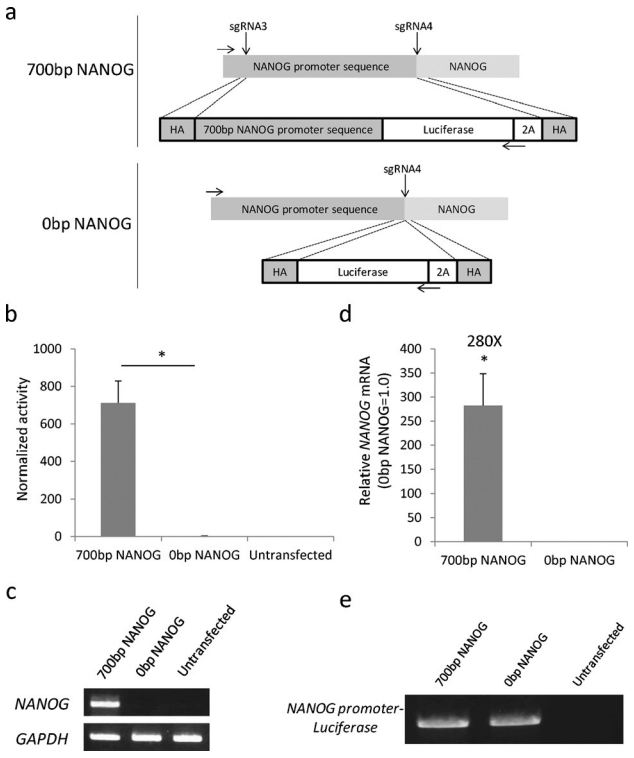

Figure 3. Activation of *NANOG* gene in HEK 293T cells by using the CRISPR/Cas9-based epigenome editing system. a) The 700-bp *NANOG* and 0-bp *NANOG* epigenome editing vectors. Arrows indicate primer sites for genomic PCR. b) Induced luciferase activity by the 700-bp *NANOG* editing vector. Error bars 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 the primers shown in (a).

domain.[34,35] This system induces rapid transcriptional activation within a few hours after blue-light irradiation, however, transcriptional activity is 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 was not able to induce cell differentiation in human pluripotent stem cells. By contrast, our epigenome editing system has been shown to activate *OLIG2* expression in NTERA-2 cells and induce their neuronal differentiation through replacement of the methylated promoter with an unmethylated one. However, our method has the limitation that it cannot be used to activate several genes simultaneously, as Konermann et al. have demonstrated with the dCas9–VP64 system.[8]

To construct vectors with an unmethylated target promoter, we extensively used the transformation-competent *E. Coli* DH5 strain (*hsdR*17(rk-,mk+), *recA*1, *relA*1, *supE*44, *thi*-1, *endA*1, *gyrA*96).[37] Although most competent strains contain three DNA methyltransferases, Dam (methylates adenine in GATC), Dcm (methylates internal cytocine in CCAGG and CCTGG), and EcoK1 (methylates adenine in AAC(N6)GTGC and GCAC(N6)GTT), none of these methyltransferases target CpG, which is recognized by the mammalian DNA methyltransferases Dnmt1, Dnmt3a, and

Dnmt3b.[38] In addition, our data show that unmethylated *OLIG2* and *NANOG* promoters from these cells induce the expression of endogenous *OLIG2* and *NANOG* mRNA as well as the luciferase gene, thus indicating that the methyltranferase activity in the *E. coli* DH5 strain does not affect our system.

As we have demonstrated, replacement of the methylated promoter with an unmethylated one is sufficient to activate the expression of target genes. However, this raised the question of how long the unmethylated status is kept in the cells. Both Dnmt3a and Dnmt3b 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 Dnmt3b,[39] it has been shown to take 11 days until a locus demethylated by using 5-aza-2-deoxycytidine (5-Aza) has been remethylated to a steady-state.[40] Data from the Human Protein Atlas (http://www.proteinatlas.org/) indicate that the expression of Dnmt3a and Dnmt3b is relatively low in HEK293T, whereas NTERA-2 strongly expresses both genes. Since we detected *OLIG2* expression in NTERA-2 7 days after transfection (Figure 2c,d), the edited promoter should remain in the active state in the cells for at least 7 days. In the case of HEK293T, we detected *OLIG2* expression in the cells 21 day after transfection (Figure S6). Thus, our epigenome editing system activates target gene expression transiently, depending on the expression level of Dnmt3a and Dnmt3b. However, it may be possible to achieve a long-lasting active state in the specific promoter by coupling our epigenome editing system with a DNA demethylase, such as the TET1 hydroxylase.[41]

The DNA demethylating drug 5-Aza is broadly used to erase DNA methylation in a genome-wide manner[42] and to activate genes that are silenced by promoter methylation. We used 5-Aza to induce *OLIG2* expression in HEK293T and NTERA-2, however, we could not detect expression in these cells (Figure S7), thus indicating that 5-Aza does not necessarily activate the expression of any silenced gene.

In summary, the results presented herein indicate that our epigenome editing system could be applied to elucidating complex transcriptional networks at the physiological level, synthesizing genetic circuits, and engineering cells.

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