**The novel CRISPR-dCas9 based DNA demethylation system**

**Abstract:** Many significant progresses have been made in the field of Ten-eleven translocation (TET) protein induced target DNA demethylation. Highly precise demethylation process is required for the efficient DNA transcription upregulation which can benefit gene expression manipulation and potential treatment for hypermethylated tumor suppressor induced cancer. Beside the pioneering TALE repeat fused TET1 and two different DNA binding zinc fingers (ZFs) fused TET2, Xu et. al introduced a more precise and easy-to-implement method to demethylate target genes by combining popular clustered regularly interspaced short palindromic repeat (CRISPR)-Cas with TET1 catalytic domain (TET1-CD). Here we introduce the basic description and discussion of the The novel CRISPR-dCas9 based DNA demethylation system. The determination of the best binding site for optimal transcription upregulation still remains a challenge. Natronobacterium gregoryi Argonaute (NgAgo) introduced by Gao et. Al can also be applied for better binding specificity and therefore elevating the efficiency of DNA demethylation system.

**Introduction**

For multi-cellular eukaryotic organism, most cells need to do through differentiation process in order to serve different purposes, which as a whole contribute to the proper function of the whole organism. This cell differentiation process requires permanent or transient memory for their own transcriptional programs. Since all the cells in an organism have nearly the same DNA sequence, this kind of transcriptional program is written as epigenetic marks including post-translational modifications of histone proteins as transient memories and DNA methylation as permanent and heritable memories [1]. Generally, DNA methylation leads to the suppression of gene transcription and consequently silences the production of corresponding protein. Normal DNA methylation is essential for the proper protein synthesis for different cells, while aberrant DNA methylation can lead to many diseases like cancer, which in this case is caused by hypermethylated tumor suppressor genes [2]. Therefore, DNA demehtylation techniques can benefit not only our understanding of gene expression regulation process by manipulation of methylation level but also potential clinical treatments for cancer.

**Recent Progress**

Many effective ways have been introduced for DNA demethylation including direct or indirect excision-repair pathway and oxidative demethylation [1]. Ten-eleven translocation (TET) family of proteins are commonly used for oxidative demethylation [3]. TET1, one of the important member of TET family, tends to bind to CpG-rich promotors at transcription start sites [3]. Ten-eleven translocation (TET) family of proteins were first identified by Tahitian et. al for their role in 5-methylcytosine (5mC) oxidation to 5-hydroxymethylcytosine (5hmC) *in vitro* [4].After this, Maeder et. al pioneered the targeted DNA demethylation of a specific site by engineering the fusion of TET1 catalytic domain and transcription activator–like effector (TALE) repeat arrays with programmable DNA-binding specificities [5]*.* This significant and novel method opened the door for more targeted DNA demethylation using genome-editing technologies because of their high specificities.Similarly, Chen et. al managed to induce DNA demethylation on a specific gene by fusing two different DNA binding zinc fingers (ZFs) as specific binding domains to TET families targeting an 18-bp sequence in the promotor [6]*.* Recently, Xu et. al introduced a more precise and easy-to-implement method to demethylate target genes by combining popular clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system introduced by Zhang’s group[7] with TET1 catalytic domain (TET1-CD)[8]. They applied the cutting-edged synergistic activation mediator (SAM) to CRIPSR-dCas9 system by engineering additional protein-interacting apatamers to sgRNA for maximum transcription upregulation[9].More precisely, the main player TET1 was engineered to be linked to dimerized MS2 bacteriophage coat protein and dCas9 protein respectively by a linker to form MS2-TET1-CD and dCas9-TET1-CD complexes[8]. A minimal hairpin aptamer was inserted to sgRNA tatraloop and stem-loop 2 for MS2-TET1-CD protein complex binding[9]. The non-hairpin part of sgRNA is responsible for dCas9-TET1-CD protein complex recruitment. Together, these two complexes recruited by the sgRNA contribute to the effective demethylation of target genes.

**Discussion**

Xu et. Al did great controls in this research. DNA methyltransferase inhibitor significantly upregulated the the transcription of the three selected genes: RANKL, MMP2 and MAGEB2, which validates hypermethylation as the main reason for transcription suppression. Dead form of TET1 greatly decreased the transcription level compared to that of original TET1-CD, indicating the requirement of deoxygenate activity for gene activation. Also, results without sgRNA were included in the compare of controls results, highlighting the specific targeting contribution based on CRISPR-dCas9 system. In order to draw the conclusion that transcription upregulation was directly resulted from targeted demethylation, bisulfite sequence was used to determine the methylation rate among many cells for each targeted loci. Moreover, the group assessed the RANKL protein expression and the corresponding signaling pathway to verify the positive functional impact of the demethylation, which eliminates the negative effect on translation and signaling process by this demethylation system. Off-target effects of this system was carefully evaluated, which turns out to be small and suitable for efficient demethylation. The target demethylation of gene RANKL in a different cell and other genes using the CRISPR-dCas9 based system was investigated to demonstrate the general applicability of this novel demethylation system.

It may be subjective and also cumbersome to design several different sgRNAs candidates targeting different sites for best unregulation results according to even more CpG sites within promotor region. For example, there is no predetermined way to select 8 target sites for the engineering of 8 sgRNAs based on about 13 CpG sites within 800bp promotor region of RANKL gene. More efficient and objective methods like computer simulation could be applied to narrow down the list of potential binding sites for optimal transcription increase. How to determine the best binding site for optimal transcription upregulation is still a challenge since it is relevant on the relative positions of multiple CpG sites, the shape of TET1-CD, the length of the tethering linker and many other potential factors.

The optimal efficacy under certain molar ratio and certain total amount of dCas9-TET1-CD and MS2-TET1-CD was determined by experimental investigation. Some explanations and maybe more investigations should be included for a better understanding of the different transcription levels under different ratios and total amount of these two complexes. For example, beside the chosen sgRNA 3 and 8, other sgRNAs could be used to investigate the relationship between transcription levels and ratios or total amount of the two complexes since it is highly possible that this relationship may vary for different target positions. As for the explanation, the timely methylation of target genes by Tet1 may be responsible for the transcription level variation under different total amount of the complexes[3] and potential transcription repression caused by dCas9 by hindering RNA polymerase machinery[10] may account for the variation under different ratios.

For minimal off-target effect, reducing the non-specific interaction can greatly reduce the chance of off-target association. For example, two variants of Cas9, which are eSpCase9 from Zhang’s group and SpCas9-HF1 from Joung’s group can be used instead of ordinary Cas9 for optimal performance[11]. Anyway, careful off-target effect evaluation is required for any research related to specific DNA binding.

Recently, a potentially more specific and efficient DNA-guided genome editing tool called Natronobacterium gregoryi Argonaute (NgAgo) was introduced by Gao et. al[12]. More promising results could be produced using this new genome editing system by mutating the DNA cleavage site of NgAgo because it shows lower off-target effect and high specificity for G,C rich sites[12]. Additionally, it does not need protospacer-adjacent motif (PAM) as Cas9 system. However, it is possible that the direct binding of NgAgo to G,C rich sites, which are potential targeted demethylation sites, can block the TET1-CD association with CpG sites.

**References**

1. Wu, S.C. and Y. Zhang, *Active DNA demethylation: many roads lead to Rome.* Nature Reviews Molecular Cell Biology, 2010. **11**(9): p. 607-620.

2. Esteller, M., *Cancer epigenomics: DNA methylomes and histone-modification maps.* Nat Rev Genet, 2007. **8**(4): p. 286-98.

3. Williams, K., et al., *TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity.* Nature, 2011. **473**(7347): p. 343-8.

4. Tahiliani, M., et al., *Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1.* Science, 2009. **324**(5929): p. 930-5.

5. Maeder, M.L., et al., *Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins.* Nature biotechnology, 2013. **31**(12): p. 1137-1142.

6. Chen, H., et al., *Induced DNA demethylation by targeting Ten-Eleven Translocation 2 to the human ICAM-1 promoter.* Nucleic Acids Res, 2014. **42**(3): p. 1563-74.

7. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems.* Science, 2013. **339**(6121): p. 819-823.

8. Xu, X., et al., *A CRISPR-based approach for targeted DNA demethylation.* Cell Discovery, 2016. **2**.

9. Konermann, S., et al., *Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex.* Nature, 2015. **517**(7536): p. 583-8.

10. Hsu, P.D., E.S. Lander, and F. Zhang, *Development and applications of CRISPR-Cas9 for genome engineering.* Cell, 2014. **157**(6): p. 1262-78.

11. Nelson, C.E. and C.A. Gersbach, *Cas9 loosens its grip on off-target sites.* Nat Biotechnol, 2016. **34**(3): p. 298-9.

12. Gao, F., et al., *DNA-guided genome editing using the Natronobacterium gregoryi Argonaute.* Nature Biotechnology, 2016.