Precise Epigenome Editing on the Stage: A Novel Approach to Modulate Gene Expression

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ABSTRACT: In the last decades, a better understanding of human pathologies has revealed that genetic alterations as well as epigenetic aberrations can be drivers of a disease or exacerbate its manifestation. The availability of customizable platforms that allow precise genomic targeting has opened the possibility to cure genetic disorders by tackling directly the origin of the disease. Indeed, tethering of different effectors to a DNA-binding moiety grants precise alterations of the genome, transcriptome, or epigenome with the aim of normalizing disease-causing aberrations. The use of designer nucleases for therapeutic genome editing is currently approaching the clinics, and safety concerns arise with respect to off-target effects. Epigenome editing might be a valuable alternative, as it does not rely on DNA double-strand breaks, one of the most deleterious form of DNA damage, to exert its function. We have recently described designer epigenome modifier (DEM), a novel platform for achieving precise epigenome editing in clinically relevant primary human cells. We discuss the efficiency of DEM and highlight their remarkable safety profile, which certainly makes this platform a valuable candidate for future clinical translation.

KEYWORDS: Precise epigenome editing, hit-and-run, DNA Methylation, Transcription-activator-like effectors

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Introduction

The information necessary to properly assemble RNA and proteins, which represent the building blocks of any living organism on Earth, is enclosed in the cellular genome. However, instructions on how this information is accessed in time and space are held in the epigenome, defined as the whole of chemical modifications of DNA and histones that eventually grant selective access to the information contained in the DNA. This is instrumental for the specification of the multitude of cell types within an organism that, besides sharing the very same DNA sequence, are specialized in distinctive functions.

In the last decades, while the contribution of genetic alterations (i.e. DNA mutations) for multiple human pathologies has been revealed, we have observed increasing awareness that epigenetic aberrations have an important role in pathogenesis of major human disorders.[1] Of particular interest is the relationship between epigenetics and cancer with a wealth of evidence suggesting a link between pathologic alteration of the epigenome and cancer progression or metastatic potential.[2] This has propelled the development of "epigenetic drugs" (epi-drugs) that alter epigenetic marks in a global way to provide a benefit to the patient.[3] While the use of epi-drugs is currently under consideration for the treatment of some forms of cancer,[4] their lack of selectivity certainly poses concerns, since the same epigenetic mark can induce opposing effects on gene expression. Indeed, for example, while DNA methylation is generally associated with gene silencing when occurring in promoter regions, it is also associated with gene expression when present in gene bodies.[5] Thereby, global changes in methylation may result in unpredictable off-target effects.

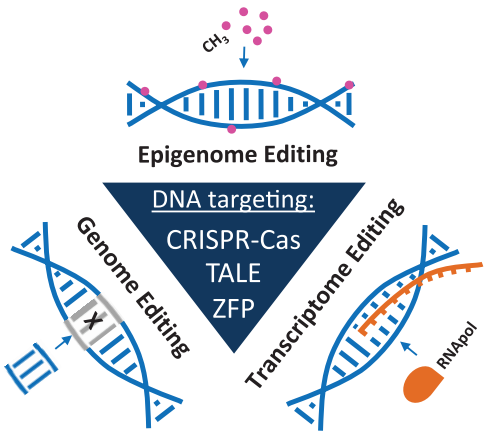

Figure 1. Precise editing using DNA-binding platforms. Schematic representation of different effector domains that can be tethered to DNA-binding platforms to achieve precise editing at different levels.

Targeted Epigenome Editing

The recent developments of DNA-targeting platforms have allowed the generation of customizable proteins capable of altering the genome or the transcriptome in a locus-specific fashion (Figure 1). This is achieve by tethering functional domains, such as nucleases or transcription modulators, to a DNA-binding moiety and has opened new avenues for gene and cell therapy. To date, designer nucleases that use DNA-binding domains (DBDs) derived from transcription activator-like effectors (TALEs) or based on the clustered regulatory interspaced short palindromic repeat (CRISPR)-Cas9 system for achieving target specificity, are approaching the clinic with the aim of establishing novel therapeutic for human immunodeficiency virus (HIV)

infection or cancer.[6] However, the keyword when thinking of using customizable effectors to establish novel therapeutics is "*safety*." This implies that the effects at off-target sites, DNA sequences that besides being different than the target site can still be bound by the used effector, have to be accurately evaluated to anticipate potential side effects of the designed therapeutics. In an ideal scenario, the selected effector has high desired activity at the target site with minimal or undetectable activity at off-target sites evaluated in a genome-wide fashion.

Even by selecting highly specific effectors, in some circumstances, targeting of multiple genes might be essential. An example is the simultaneous inactivation of the two HIV co-receptors (i.e. C-C chemokine receptor type 5 [*CCR5*] and C-X-C chemokine receptor type 4 [*CXCR4*]) to render the target cells, such as T lymphocytes, broadly resistant to infection from several HIV types. In principle, this can be achieved by concomitant delivering of two designer nucleases for multiplex gene inactivation as attempted previously.[7] However, while promising, an approach like this will certainly result in deleterious genomic aberrations, such as translocations,[8] as result of the multiple DNA breaks generated by the designer nucleases used and occurring in the same cell at the same time. This underlies the need for alternative strategies for multiplex gene inactivation, and precise epigenome editing might represent a valuable opportunity.

A Multidomain System for Gene Silencing

As we have seen for genome or transcriptome editing, the same principle has been exploited to modify the epigenome in a site-specific manner (Figure 1), thus overcoming one of the major hurdles associated with the use of epi-drugs.[9] We have recently established designer epigenome modifier (DEM), a novel platform to control gene expression by locus-specific alteration of the epigenome.[10] Designer epigenome modifiers are capable of specifically targeting single sites in a given genome, taking advantage of the highly specific DBD derived from bacterial TALEs. The epigenome editing function is provided by the direct fusion of key epigenetic factors to the TALE-DBD, thereby ensuring specific changes of desired epigenetic marks at the site of DBD binding. In DEM, we promote deposition of two epigenetic marks typically associated with gene silencing, namely DNA methylation and tri-methylation of histone 3 on lysine 9 (H3K9me3). This is achieved in a site-specific manner through the direct fusion to the TALE-DBD of the catalytic domain of the *de novo* methyltransferase DNMT3A and the Krüppel-associated box (KRAB) domain, respectively. To further stimulate the on-target deposition of *de novo* DNA methylation, we included the C-terminal domain of the DNA methyltransferase 3-like (Dnmt3L) in the DEM structure, which does not have catalytic activity *per se* but directly interacts with DNMT3A and enhances its function.[11] We have used this platform to simultaneously silence two genes which are involved in early stages of HIV infection, specifically the *CCR5* and the *CXCR4*. The corresponding proteins are co-receptors that allow for HIV entry into the target cells, and, through their silencing, we aim to induce resistance to HIV infection in transplantable primary human cells. The activity of DEMs was first assessed on a surrogate reporter cell line where the expression of an enhanced green fluorescent protein (EGFP) was driven by a chimeric promoter composed of a minimal cytomegalovirus (CMV) promoter and a *CCR5* proximal promoter fragment including the binding sites of *CCR5*-specific DEMs we generated. In this context, the DEMs were capable of rapidly silencing the reporter gene, with maximal activity reached within 6 days post-delivery and in a sustained manner for a period of up to 2 months in culture. Interestingly and unexpectedly, efficiency was not related to the duration of DEM expression. Indeed, ensuring long-term expression via DNA transfection of a plasmid containing a DEM expression cassette resulted in only mild green fluorescent protein (GFP) silencing. On the other hand, short-term expression mediated by transfecting *in vitro*-transcribed mRNA encoding for the desired DEM led to a dramatic improvement of DEM's activity. This suggests that the efficiency observed might result from the on-target assembly of a multi-component complex, which is promoted by DEM binding and drives sustained gene silencing through deposition of *de novo* DNA methylation and H3K9me3. However, since catalytically inactive DEMs were unable to drive EGFP silencing, we hypothesized that direct on-target catalytic activity combined with the recruitment of endogenous epigenetic factors is essential for achieving sustained gene silencing. Importantly, the simultaneous presence on-site of the different epigenetic factors is crucial to achieve high levels of gene silencing. Tethering the DNMT3A-3L fusion and the KRAB on neighboring sites, by coupling the two epigenetic factors to different DBDs, resulted in significantly lower gene silencing ability. Even more alarming was the complete loss of efficacy in primary human cells, where the delivery of multiple epigenetic effectors was ineffective as compared to the all-in-one DEM. This can be explained by the local chromatin context that may impede the correct assembly of the silencing machinery when the different components are not simultaneously present at the target site. In contrast with our findings, previous reports have shown that the fusion of DNMT3A, DNMT3L, and KRAB epigenetic factors to three different DBDs resulted in efficient and sustained gene silencing upon simultaneous delivery.[12] While the authors of this study did not provide evidence that their strategy could be used to control endogenous gene expression in primary human cells, it is certainly interesting to understand the subtle differences between the two systems that account for the discrepancy observed in our side-by-side comparison. We believe, however, that an all-in-one architecture, as for DEMs, facilitates the applicability of epigenome editors in primary human cells, which are typically sensitive to overt manipulation. While on one hand, the large size of all-in-one DEM certainly poses concern for vectorization, manufacturing of a single mRNA molecule to deliver DEM into primary hematopoietic stem cells or T lymphocytes is certainly easier and cost-effective.

Safety of Epigenome Editing

An important aspect in genome as well as in epigenome editing is to keep off-target effects at minimum.[13] In our work, we have widely characterized the promiscuous activity of DEMs at off-target sites and have highlighted their remarkable specificity. Since we assumed that most of the *de novo* methylation induced by DEMs at off-target sites would be silent (unless occurring in cis-regulatory regions or gene bodies), we decided to investigate whether expression of DEM in target cells for a limited period of time (as consequence of mRNA delivery) was sufficient to drive measurable phenotypic alterations, which can be a result of off-target binding. Importantly, we performed such analysis in clinically relevant primary human cells to avoid confounding effects that may arise from non-physiological conditions affecting immortalized cell lines. We first analyzed the overall impact of DEMs expression on the cellular transcriptome via RNA-seq analysis. While, as expected, the target gene (i.e. *CCR5*) was significantly downregulated, we found, in addition, 83 genes differentially expressed as compared to cells undergoing similar manipulations but receiving an inactive DEM. Subsequently, we searched *in silico* for potential off-target DEM-binding sites in a 10 kb window from the transcription start sites of the 83 deregulated transcripts identified. By allowing up to three mismatches as compared to the on-target site, we did not retrieve any potential off-target site that could account for non-specific DEM binding and subsequent alteration of gene expression. We thereby concluded that the changes in gene expression were not due to DEM off-target activity. We next hypothesized that off-target DNA methylation might have impacted on chromatin structure and thereby could have reduced chromatin accessibility at off-target sites. To address this aspect, we performed whole genome assay for transposase-accessible chromatin (ATAC-seq). This assay uses a transposase to tag all the regions of accessible chromatin that can be subsequently mapped via next generation sequencing. Again, as compared to the same control samples, we validated lower chromatin accessibility at the on-target site in the *CCR5* promoter and, additionally, at 324 chromatin regions. Again, we searched for potential DEM off-target-binding sites in a 10 kb window from these regions and found three sites, each harboring three mismatches that could potentially be involved in the lower chromatin accessibility measured at these sites. However, also in this case, we could exclude direct DEM off-target effect for two reasons: (1) the distance from the corresponding region with lower chromatin accessibility is too large to account for direct effects, and (2) in all three cases, nearby transcripts were not altered in their expression levels. Importantly, the target gene *CCR5* could be retrieved with both techniques, validating the assumption that if measurable phenotypical off-target effects occurred, they would have been emphasized by both assays used. Ultimately, we analyzed *de novo* methylation at the top-10 *in silico*-predicted off-target sites. We found increased methylation at a single site suggesting that off-target binding occurs, as it is the case for all other classes of designer effectors. However, we confirmed that, since epigenome editing does not rely on the introduction of DNA breaks, it is intrinsically safer, as most of the off-target effects are silent, as they occur in intergenic or non-regulatory regions. It is important to note, though, that with our analysis, we could not account for long-distance effect that may arise by the tridimensional chromatin structure, which might bring unrelated genomic regions in close proximity to on-target as well as of off-target binding sites. It will be certainly interesting and critical to assess in the future whether the alterations highlighted by RNA-seq and ATAC-seq may be due to this phenomenon.

Concluding Remarks

Targeted epigenome editing represents today a promising strategy to stably control gene expression in cells that do not undergo massive chromatin remodeling. Our all-in-one architecture enables safe and cost-effective transfer of this concept in clinically relevant primary human cells, thereby opening new avenues for the exploitation of epigenome editing to treat human disorders. We are today in an era of great changes in the fields of cell and gene therapy, and novel technologies open new opportunities for scientists, physicians, and patients. The implementation of assays to properly monitor the impact of epigenome editors on target cells will certainly boost the use of this novel platform for therapeutic applications.

Author Contributions

CM wrote the manuscript.

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