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Can genome engineering be used to target cancer-associated enhancers?

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Abstract

Transcriptional misregulation is involved in the development of many diseases, especially neoplastic transformation. Distal regulatory elements, such as enhancers, play a major role in specifying cell-specific transcription patterns in both normal and diseased tissues, suggesting that enhancers may be prime targets for therapeutic intervention. By focusing on modulating gene regulation mediated by cell type-specific enhancers, there is hope that normal epigenetic patterning in an affected tissue could be restored with fewer side effects than observed with treatments employing relatively nonspecific inhibitors such as epigenetic drugs. New methods employing genomic nucleases and site-specific epigenetic regulators targeted to specific genomic regions, using either artificial DNA-binding proteins or RNA–DNA interactions, may allow precise genome engineering at enhancers. However, this field is still in its infancy and further refinements that increase specificity and efficiency are clearly required.

Keywords

CRISPRs; DNA methylation; enhancers; epigenetic therapy; gene expression; genome engineering; genomic nuclease; histone modifications; TALENs; ZFNs

Enhancers as therapeutic targets

Transcriptional misregulation is involved in the development of many diseases, especially neoplastic transformation [1]. Recent technological breakthroughs have enabled genome-wide analyses of the locations and activities of transcriptional regulatory elements in normal and cancer cells. Such studies have revealed alterations in chromatin structure during neoplastic transformation due to epigenetic changes such as DNA methylation, histone modifications and nucleosome positioning at regulatory elements that are proximal (promoters) or distal (enhancers) to the transcription start sites of genes [2,3]. Although there is no strict definition of an enhancer, they are often classified as short regulatory

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elements that can activate genes in an orientation-independent manner at long distances, often bypassing the nearest gene [4]. Specific modifications that characterize enhancers include a low level of DNA methylation and high levels of modified histones, such as histone H3 acetylated on lysine 27 (H3K27Ac) or monomethylated on lysine 4 (H3K4me1) [5]. Analysis of these epigenetic characteristics on a genome-wide scale, using techniques such as whole genome bisulfite sequencing (to monitor DNA methylation) and ChIP-seq (to monitor histone modifications), has identified more than 400,000 enhancers in the human genome and has predicted the existence of over 1 million distal regulatory elements [6].

Enhancers are bound by complexes of transcription factors and coactivators, which aid in guiding enhancers to gene promoters (via looping) and in recruiting and/or activating RNA polymerase II (Pol II) [7]. Evidence suggests that changes in epigenetic states that promote transcription factor binding at enhancers may be more closely related to changes in gene expression than are changes in promoter epigenetic states [8,9]. Importantly, large-scale studies have revealed that enhancers are one of the most dynamically utilized regulatory elements [10], suggesting that they play a major role in specifying cell-specific transcription patterns in both normal and diseased tissues. Furthermore, genome-wide association studies have recently revealed many genetic variants that are statistically associated with disease, but are far from any protein-coding genes [11]. Jia *et al.* showed that one such region associated with breast, colorectal and prostate cancers harbored multiple enhancers with sequence variants that aided the binding and activity of transcription factors known to collaborate in these cancers [12,13]. An array of cancers display enhancer clusters that associate with important lineage-specific and tumor genes, possibly orchestrating tumor-specific transcriptional programs [14,15]. The recent discovery that Pol II binds active enhancers and transcribes enhancer RNAs with similar characteristics to promoter-derived mRNAs has opened a new field of functional studies on enhancers [16]; enhancer RNA expression correlates with H3K27ac levels, can direct chromatin remodeling, contributes to gene activation and can even drive tumorigenesis [17,18]. Taken together, such studies suggest that enhancers may be prime targets for therapeutic intervention.

Epigenetic inhibitors have been developed to affect DNA methylation [19], histone acetylation status [20-22] or the function of enhancers [14]. However, such agents have a broad specificity and many off-target effects. Because enhancers are cell type specific, targeting a single enhancer could allow the cell type-specific regulation of a gene whose misregulation drives tumorigenesis (Figure 1). For example, using standard homologous recombination, Sur *et al.* deleted a 1740 bp region that lies approximately 335 kb upstream from the murine *Myc* gene [23]. This resulted in decreased expression of MYC in the colon, but not the duodenum, and a reduction in the number of polyps per animal in a mouse model for colon cancer. Such results suggest that specific inactivation of tumor-specific enhancers or reactivation of enhancers lost in tumors may result in a reduced tumorigenic phenotype, with fewer side effects than treatment with drugs that affect genome-wide levels of DNA methylation or histone acetylation. As established tumors are often heterogeneous, with epigenetically distinct cell subpopulations, simultaneous targeting of multiple enhancers in a patient may present an attractive therapeutic option. Two new methods that could be used to perform precise genome engineering at specific enhancers are described below.

Site-specific DNA targeting approaches

To bind and manipulate a specific genomic locus, nucleases or transcriptional regulatory domains must be recruited to the target site with efficacy and specificity. The earliest genomic targeting proteins were based on zinc finger (ZF) DNA-binding domains. Many artificial ZF proteins employ six fingers and, because each finger domain recognizes three nucleotides, they are designed to recognize a specific 18-nucleotide stretch in the genome. However, a recent large-scale screen of modular ZF assemblies found that 70% fail to bind their designed target sequence [26], demonstrating that our understanding of the complex rules governing efficient ZF–DNA interactions is far from complete. Furthermore, ZF assemblies are difficult to create and have a limited targeting capability owing to the nature of ZF–DNA interaction requirements, and thus are being rapidly supplanted by two newer technologies: transcription activator-like effectors (TALEs) and clustered regularly interspaced short palindromic repeats (CRISPRs).

TALEs are genomic targeting platforms based on bacterial DNA-binding domains [27]. TALE DNA-binding domains are composed of a series of tandem repeats, each of which bind a single nucleotide [28,29], allowing the targeting of a wider percentage of the genome than a ZF. These constructs are also easier to clone than ZFs, using an archive of premade domains. CRISPR, the newest genomic targeting platform, utilizes a short, specific guide RNA (gRNA) that brings a bacterial-derived Cas9 protein to a complementary genomic sequence [30]. By simply changing the 5' end of a gRNA, the Cas9 protein can be directed to virtually any locus in the genome, greatly simplifying the logistics of targeting new genomic sites.

Each of these three platforms can be used to direct dsDNA breaks; see [31] for detailed descriptions of each targeting platform. To create a targetable nuclease, the cleavage domain of the nonspecific restriction enzyme, FokI, can be appended to ZF or TALE domains, creating ZF nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) (Figure 2). FokI has been engineered to work as an obligate heterodimer to reduce off-target effects [32]. Therefore, two ZFNs or two TALENs are designed to recognize adjacent sites on opposing DNA strands so that FokI dimerization can occur and create a double-strand cleavage in the DNA. The Cas9 protein in the CRISPR system is a nuclease and thus does not require further engineering for use in this methodology. Excision of a large genomic region can occur if two pairs of nucleases, each having target sequences on either side of the region, are employed [33]. In this approach, the 5' and 3' nucleases are introduced into cells simultaneously and the cells are then screened for deletion by PCR. If a donor oligonucleotide is supplied, the targeted region (e.g., a disease-related allele) may be replaced, rather than deleted. TALENs and CRISPRs offer the advantages of higher cutting efficiency and simpler assembly than ZFNs. Unlike ZFNs and TALENs, Cas9 of the CRISPR system can cleave DNA as a monomer, which has the advantage of easier design and cloning, but a disadvantage of more potential off-target effects.

Site-specific alteration of regulatory elements can also be achieved using epigenetic toggle switches, which are ZF-, TALE- or CRISPR-targeting platforms with an epigenetic-modifying domain in place of the nuclease moiety. Epigenetic-modifying domains can either

act through direct enzymatic activity (e.g., DNA methyltransferase, TET or G9a) or indirectly by recruiting epigenetic enzymes (e.g., KRAB, VP64 or p65AD). When the CRISPR platform is used for such experiments, a modified Cas9 is employed; the modified Cas9 is catalytically inactive but can still interact with a gRNA, allowing genomic targeting without cutting the DNA [34]; this modified protein is termed dCas9.

In general, active regulatory elements display low levels of DNA methylation and high levels of acetylated histones. Therefore, increasing the levels of DNA methylation or decreasing the levels of acetylated histones at regulatory elements is the goal when employing epigenetic toggle switches to repress an enhancer. Because our current understanding of the relationship between different epigenetic modifications and gene expression remains largely associative, targeted epigenetic manipulation can provide mechanistic insights into the effects of modulating a particular epigenetic mark on gene expression. Thus, we note that these current studies will be very informative to the field for the future design of optimized epigenetic toggle switches.

Genetic manipulation of regulatory elements

One potential therapeutic approach to target a tumor-specific enhancer would be to precisely delete the regulatory element (or alter a specific transcription factor binding site or SNP within the enhancer) from the genome of the tumor cells. Alternatively, genomic nucleases can be used to exchange a genomic segment with one containing an alternative allele or a mutated transcription factor binding site. The use of site-specific genomic nucleases has advanced rapidly, leading the journal *Nature Methods* to award targetable nucleases the Method of the Year in 2011 [35]. However, the concept of deleting entire regulatory elements or functional sequences within regulatory elements is quite new; therefore, very few examples have been published. Fortunately, targeted deletions of up to 15 mega-bases (much larger than a typical enhancer) have been reported in human cell lines [33]; thus, this approach is quite feasible. One example of employing genomic nucleases to target an enhancer comes from Webster *et al.*, who used TALENs to delete 7 bp (corresponding to a binding site for the transcription factor MITF) from the middle of a human melanocyte-specific enhancer that is 63 kb away from the promoter of the *MET* gene [36]. This small deletion resulted in reduced levels of H3K27Ac at the enhancer and decreased looping from the enhancer to the *MET* promoter, preventing induction of the *MET* gene. A second study used TALENs to alter a SNP within a distal element 44 kb upstream of the *BUB1B* gene [37]. Using a two-step method, Ochiai *et al.* introduced a mosaic variegated aneuploidy syndrome-associated candidate SNP in place of the common allele, and observed an approximately twofold reduction in *BUB1B* expression (consistent with the expression difference between diseased and normal cells) and increased disease phenotype.

Epigenetic modification of regulatory elements

A second method to target a specific regulatory element is to epigenetically alter its function. Active enhancers or promoters are marked by low levels of DNA methylation, so increasing DNA methylation should, in principle, inactivate an enhancer or promoter. Conversely, decreasing DNA methylation should reactivate a silenced enhancer or promoter.

Using this rationale, one study employed six ZFs linked to a DNA methyltransferase domain and achieved DNA methylation and modest repression of the *Maspin* and *SOX2* promoters [38]. Interestingly, these promoters were stably repressed even upon withdrawal of the ZF-DNA methyltransferase, suggesting that a long-term repression can be achieved by transient expression of an epigenetic toggle switch. Examples in which methylated promoters have been epigenetically reactivated come from studies using TALE or ZF platforms linked to the active domains of the DNA demethylases TET1 and TET2. Introduction of TALE-TET1 toggle switches resulted in reduced DNA methylation within 30–200 bp of the binding sites at some targeted promoters [39]. Unfortunately, only a subset of the promoters that reduced methylation showed increased gene expression, perhaps owing to the fact that the cytosines that responded to the demethylase were not the ones involved in promoter silencing and/or that a required activating transcription factor was absent from the cells. Similarly, using the TET2 DNA demethylase fused to a ZF DNA-binding domain, Chen *et al.* observed modest losses of DNA methylation and small changes in gene expression at the target *ICAM1* promoter [40].

Altering the levels of histone modifications can also affect the activity of a regulatory element. Mendenhall *et al.* attached an LSD domain, which catalyzes the removal of H3K4 methylation, to TALE domains and targeted a series of enhancers [41], achieving modest changes in histone marks at many of the targeted enhancers and modest gene expression changes at some nearby genes. In an attempt to obtain more significant effects on gene expression, they targeted three intronic enhancers of the *ZFPM2* gene (within 50 kb of each other). Unfortunately, simultaneous targeting of the three enhancers did not cause a greater reduction in gene expression. Stolzenburg *et al.* fused a domain that recruits a repressive histone methyltransferase to a ZF domain and showed repression of the *SOX2* promoter and subsequent effects on cell growth and tumorigenicity [42]. To achieve the opposite effect, in other words, gene activation, Perez-Pinera *et al.* targeted endogenous promoters with TALE-VP64 or dCas9-VP64 constructs [34,43]; VP64 is a tetramerized domain of the VP16 viral transactivator that functions, in part, by recruiting histone acetyltransferases. Notably, they found that multiple TALE-VP64 constructs or multiple gRNAs could synergistically activate transcription when targeted to a promoter. Gilbert *et al.* also used the CRISPR system for gene regulation, achieving synergistic activation (with VP64 and p65AD) and repression (using a KRAB domain) when targeting reporter constructs and endogenous human promoters and enhancers [44]. The commonly observed synergistic activity of multiple CRISPR toggle switches, along with the ease of cloning gRNAs, is likely to bring the CRISPR system to the forefront for epigenetic modification of regulatory elements.

Technological challenges

Attempts to use genomic nucleases or epigenomic toggle switches to target cancer-specific enhancers are in their initial stages. Although these approaches do seem to offer a uniquely specific way to affect expression of oncogenes or tumor suppressor genes in a cell type-specific manner, there are issues related to efficacy and specificity that need to be addressed as the field moves forward.

In the studies performed to date, a low efficiency of deletion and modest effects on gene expression have been observed. In an attempt to identify highly effective epigenetic modulators, Konermann *et al.* linked TALEs to a large set of domains that have either activating or repressive activities [45]. Most of these toggle switches worked to some degree, but the levels of activation and repression were fairly modest. To date, no strict rules have been forthcoming on how to predict the effectiveness of the nucleases or toggle switches, but it is possible that overall chromatin structure may be involved. For example, heavily methylated, nucleosome-dense regulatory elements might be difficult to access [39]. However, the presence of a DNase hypersensitive site (indicative of open chromatin) is not a strong predictor of the activity of an epigenetic toggle switch [34]. One study has suggested that the interaction between the gRNA and the Cas9 protein is the main determinant of effectiveness in the CRISPR system [46]; if so, perhaps better design of the gRNAs will increase the utility of the toggle switches. On a positive note, based on the few studies that followed up the gene expression experiments with cell growth or tumorigenicity assays, it appears that modest effects on transcription can lead to significant changes in cellular phenotypes [23,38,42].

The problem of potential off-target effects due to relaxed specificity of the DNA-binding domains of TALEs and ZFs, and mismatched interactions between gRNAs and genomic sequences must be considered for both genomic nucleases and epigenetic toggle switches [47-50]. For example, many cellular ZF proteins are able to bind DNA targets with only two or three contiguous fingers, suggesting that the theoretical specificity of six tandem ZFs (a unique 18 bp motif) may not be achieved in practice [51,52]. Furthermore, each finger may experience target site overlap, where a given finger may interact with four or five nucleotides (rather than the expected triplet) or may be affected by neighboring protein side chains, further complicating predictable targeting [53,54]. The commonly used CRISPR nuclease functions as a monomer, which could result in more off-target cutting than with the dimeric TALENs and ZFNs.

Unfortunately, the *in vivo* binding specificity of genomic nucleases and epigenetic toggles switches has not been well documented to date. Most studies have tested specificity of cutting or modification at the target site and perhaps at one or two predicted off-target sites, but have not performed whole genome analyses [49,55]. However, a few studies have begun to examine genome-wide specificity. Recent genome-wide analyses suggest that off-target binding can be extensive with ZF-based toggle switches [56]. By contrast, Mendenhall *et al.* used ChIP-seq to show that the top-ranked TALE-binding site was at the regulatory element to which the TALE was designed [41]. Recent progress in TALE design has arisen from a refined understanding of the TALE repeat structure [57], and by random mutagenesis and cyclic selection of TALEs in yeast [58]; such improvements may aid in designing highly specific TALENs and TALE toggle switches. In the first genome-wide characterization of dCas9 binding (using four different gRNAs) by ChIP-seq, Wu *et al.* reported tens to thousands of dCas9-binding sites, where partial gRNA–DNA pairing frequently occurs at regions of open chromatin [59]. It is important to consider that off-target binding by ZF, TALE or Cas9 may not always lead to off-target activity. For example, Perez-Pinera and

colleagues tested the specificity of dCas9 fused to a VP64 transactivation domain by RNA-seq and only the target gene showed significant increased expression [34].

Improvements in CRISPR specificity and efficacy are being made through variations in gRNA length, gRNA and Cas9 titration, improved gRNA design algorithms and the use of Cas9 proteins (from different bacterial strains) that have a more stringent targeting sequence requirement [49,60-64]. Furthermore, a modified version of the CRISPR system that may provide greater functional specificity has been developed. This system requires two gRNAs to recruit modified Cas9 proteins (each of which can nick the DNA); the single nicks will be repaired at the off-target sites, but the double nicks at the target sites will become double-strand breaks [65]. Specificity in the toggle switch platforms may come from the commonly observed highly synergistic activation when multiple effector domains are recruited to the same promoter [66]; it is unlikely that different DNA-binding domains or gRNAs all designed to target a single regulatory element will cluster elsewhere on the genome.

Conclusion

Owing to recent technological advances, it now seems possible that precision editing of the human genome may soon be included as one component of personalized medicine. By focusing on modulating gene regulation mediated by cell type-specific enhancers, there is hope that normal epigenetic patterning in an affected tissue can be restored by epigenetic toggle switches with fewer side effects than observed with treatments employing relatively nonspecific inhibitors, such as epigenetic drugs. However, this field is still in its infancy, and further refinements that increase specificity and efficiency are clearly required.

Future perspective

It is likely that genomic engineering will be favorably considered for multiple types of clinical use in the near future. In fact, ZFNs are already in clinical trials (NCT-00842634, NCT-01044654 and NCT-01252641). However, these trials, and similar studies using mouse models [67], involve genome engineering of isolated blood cells that are then reintroduced into the patient (or mouse). This is experimentally much simpler than directing the genomic nucleases and epigenetic toggle switches to every nucleus of a solid tumor, which may have regions of poor vascularization that present a pharmacokinetic challenge, allowing survival of cell populations with metastatic potential. However, sophisticated methods to target tumors are being developed. For example, Lara *et al.* used the chemical ligand anisamide to coat the surface of liposome–protamine–RNA nanoparticles, providing target specificity for sigma receptors, which are over-expressed in a large number of human tumors. The encapsulated RNA encoded a ZF-VP64 fusion targeting the epigenetically silenced promoter of the murine tumor suppressor, Maspin. Intravenous injection in mice with ovarian carcinoma resulted in Maspin activation and a reduction in tumor burden [68]. Such nanocarriers can be designed to deliver chemically modified RNAs to the targeted tumor site with high efficiency and specificity.

Enhancements to such methods could include the fusion of the nuclease or toggle switch protein to cell-penetrating peptides [69] or the activation of the proteins in a highly localized

manner [70]. For example, a ZF-KRAB repression toggle switch harboring a cell-penetrating peptide was generated to regulate expression of the epigenetically silenced Ube3a gene in Angelman syndrome, a rare neurologic disorder. Upon interperitoneal injection in a mouse model of the disease, the purified toggle switch was able to cross the blood–brain barrier, enter the neurons, repress an antisense inhibitory mechanism and consequently activate expression of Ube3a [Segal DJ, Pers. Comm.]. Furthermore, Konermann *et al.* developed a light-inducible system in which a TALE is fused to light-sensitive cryptochrome 2; blue light stimulation allows interaction of the TALE with an epigenetic-modifying domain. Using this system, they achieved reversible modulation of mammalian endogenous gene expression and targeted epigenetic chromatin modifications in mouse brains [45].

In summary, although not yet adapted for clinical use, we anticipate that a combination of increased efficiency of the functional domains and specificity of the targeting platforms, along with the inclusion of tumor-specific homing or activation domains, will allow the technology of enhancer engineering to complement current epigenetic therapeutic approaches.

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Executive summary

Enhancers as potential therapeutic targets

- Recent epigenomic studies have revealed over 400,000 enhancers in the human genome.
- Enhancers are marked by transcription factor binding sites, high H3K27Ac, high H3K4me1 and low DNA methylation.
- Small molecules targeting the epigenome have shown promise in their action on enhancers, but their lack of specificity is a concern.
- Epigenetic modulators that can specifically target enhancers may increase the therapeutic index.

Using site-specific genomic nucleases to delete enhancers

- The most common targeted genomic nucleases consist of a sequence-specific DNA- or RNA-binding domain and a nuclease domain.
- Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are DNA-binding proteins that each function in pairs, where neighboring binding sites allow heterodimerization of the FokI nuclease to cleave DNA.
- ZFNs were the first to be developed, but owing to their limited targeting capabilities have been quickly supplanted by TALENs and the Cas9-based clustered regularly interspaced short palindromic repeat (CRISPR) system.
- The RNA-guided CRISPR system provides a rapid means of specifically targeting the Cas9 nuclease to the human genome.

Epigenetically modifying enhancers

- To create epigenetic toggle switches, epigenetic effector domains replace the nuclease domains fused to ZFs or TALEs, and Cas9 is modified to bind DNA without cutting.
- Epigenetic effector domains fused to ZF, TALE or Cas9 targeting platforms can directly or indirectly modulate DNA methylation, histone methylation or histone acetylation.

Technological challenges

- There are currently few examples in which enhancers have been targeted using nucleases or epigenetic toggle switches.
- Chromatin structure may partially explain the observed limited efficacy of epigenetic toggle switches.
- Off-target binding is a concern for all genomic nucleases and epigenetic toggle switches. However, few studies have characterized genomic binding profiles for genomic nucleases or epigenetic toggle switches.

- Technological improvements are being made as more data arise to inform design principles.

Conclusion

- Genomic nucleases and epigenetic toggle switches, although in the early phase of development, show great promise for future clinical applications.

Future perspective

- Advanced targeting platforms may further increase the therapeutic utility of targeting cell type-specific enhancers.

Figure 1. Cell type-specific enhancers near the *MYC* oncogene

The *MYC* gene is upregulated in many cancers and a large region upstream of the *MYC* gene harbors many SNPs that have been linked to an increased risk for several different cancers [24,25]. Regulatory elements driving *MYC* expression are very cell type specific. The distinct ChIP-seq patterns are shown for the enhancer mark H3K27Ac in HCT116 colon cancer cells versus PANC1 pancreatic cancer cells, and a variety of different normal and tumor cells (ENCODE Consortium data available via the UCSC genome browser [6]). Inactivation of one specific enhancer (indicated by the arrow) may reduce *MYC* expression in colon cells but not in pancreatic cells.

Figure 2. Toolkits for genomic and epigenomic engineering

Targeting specificity can be provided by the DNA-binding domains of the ZFs and TALE domains or by the guide RNAs for the CRISPR platform. When used for genomic modifications, a FokI nuclease domain is attached to the ZF or TALE DNA-binding domains (this requires heterodimer formation for activity); the Cas9 protein serves as the nuclease for the CRISPR system. To create epigenetic toggle switches, a transcriptional repression domain or a transcriptional activation domain can be fused to the ZFs, TALEs or the Cas9 protein. Transcriptional repression domains and transcriptional activation domains can drive changes in histone modifications either directly (with enzymatic activity) or indirectly (through recruitment of epigenetic-modifying enzymes). Similarly, DNA methyltransferase or TET domains can be used to modulate DNA methylation at target sites. When used for epigenetic modification, Cas9 must be catalytically inactive (indicated as dCas9).

CRISPR: Clustered regularly interspaced short; palindromic repeat; TALE: Transcription activator-like effector; ZF: Zinc finger.