CHAPTER

13 The emerging field of epigenetic editing: implication for translational purposes for diseases with developmental origin

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Summary of the epigenetic editing field

Epigenetic writers, erasers, and their small molecule inhibitors

DNA methylation is established by the de novo methyl transferases DNMT3a and DNMT3b and maintained across cell division by DNMT1, which preferentially recognizes hemimethylated DNA over nonmethylated DNA.[1] DNA methylation can be removed by ten−eleven translocation methylcytosine dioxygenases (TETs) including TET1, TET2, and TET3.[2] Histone modifications are catalyzed by different enzymes. Various histone acetyl transferases (HATs), and histone deacetylases (HDAC) catalyze or remove acetylation on lysine. Histone methyl transferases (HMTs) and demethylase catalyze or remove methylation on lysine, and the protein arginine methyltransferases (PRMT) catalyze arginine methylation on the histone tail. Small molecule inhibitors are chemical compounds screened from small molecule libraries that interfere with specific biological processes. Some small molecule inhibitors target epigenetic processes and are used in basic research as well as in therapy development. The targets of these inhibitors are usually writers or erasers of the epigenetic marks.

DNA hypomethylating agents such as DNA methyltransferase inhibitors (DNMTi) reduce DNA methylation and have been used in anticancer treatment.

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5-Azacytidine (5-azaC, or 5-aza) and 5-Aza-2′-deoxycytidine (5-azadC) are two representative DNMTis used in the clinical setting.[3] Small molecule inhibitors of histone modification are also used in cancer treatment, e.g., HDAC inhibitors. However, these small molecule inhibitors are not locus specific and can potentially lead to side effects. Thus, the dosage of epigenetic drugs based on small molecule inhibitors is limited, which might compromise their potency. Therefore, the development of specific epigenetic editing tools is important for translational purposes.

Targeted epigenetic editing

Targeted DNA methylation editing

Targeted DNA methylation editing can be achieved by fusing DNMT or TET with DNA-binding proteins such as zinc finger proteins (ZF), transcription activator-like effectors (TALEs), and catalytically dead Cas9 (dCas9).

Targeted gene activation by erasing DNA methylation

The catalytic domain of TET1 or TET2 can be targeted to erase DNA methylation. To increase the efficacy of demethylation, TET1 can be tethered with DNA repair proteins, since DNA repair follows the oxidization of 5-mC to ultimately remove DNA methylation.[4] In addition, 5-mC can be directly excised by a protein found in plants, repressor of silencing 1 (Ros1). dCas9-Ros1 was used for excising DNA methylation and gene reactivation.[5]

Targeted gene repression by inducing DNA methylation

DNMT3A and DNMT3B establish de novo DNA methylation in a replication-independent manner, and DNMT3A is more catalytically active than DNMT3B.[6] Thus, human DNMT3A is the most frequently used effector protein for induction of DNA methylation.[7] Since DNMT3A is a large molecule (130 kDa), only its catalytic domain is used as the effector. The catalytic domain of DNMT3A has been fused with ZF, TALE, and dCas9 in previous studies. Additionally, the potency of DNMT3A-based targeted DNA methylation can be amplified by fusing DNMT3A with DNMT3L,[8] which stimulates the activity of DNMT3A. Besides DNMT3A, other DNA methyltransferases, including DNMT1 and MIWI2,[9] can also be fused with DNA-binding proteins to mediate targeted DNA methylation, but are less frequently used than DNMT3A.

Targeted histone modification editing

Targeted gene activation by editing histone modification

The first generation of targeted gene activation was achieved through fusing DNA-binding proteins with protein factors associated with transcriptional activation, e.g., VP16 or p65. These factors were later demonstrated to recruit enzymes that catalyze active histone marks. VP16 is a herpes virus protein that functions as a transcriptional activator.[10] VP64 is a tetramer of VP16, constructed for more potent transcriptional activation.[11]

The mechanism of VP64-mediated activation was then found to be related to histone modifications, primarily acetylation of histone H3 at lysine 27 (H3K27Ac).[12] H3K27Ac is an active enhancer mark[13] and is required for enhancer activity.[14] Acetylation of lysine on the histone tail reduces the positive charge on the lysine side chain and thus loosens its interaction with negatively charged DNA, thus making the DNA more accessible to transcription factors.

Later, it was found that H3K27Ac is directly catalyzed by p300, as shown by genetic deletion[15] or pharmacologically inhibition of p300.[14] The discovery of enzymes that catalyze specific histone marks gave rise to the second generation of targeted transcriptional activation tools. As an example, p300 was also targeted to activate enhancers, and dCas9-p300 activates the distal enhancer that is irresponsive to editing by dCas9-VP64.[16]

P65, another transcriptional activator used in first-generation epigenetic editing, is an NF-kappa B transcription factor with strong transcriptional activation capability.[17] Targeting p65-TetR (tet repressor) to a sequence with TetO (tet operator) insertion is associated with acetylation of histone H3 at lysine 9 (H3K9ac), although the effect on transcriptional activation was less potent than VP16.[18]

In addition to acetylation of the lysine side chain, methylation of lysine residue 4 on histone H3 (H3K4me3) at transcription start sites (TSS) is also associated with transcriptional activation. PRDM9 is a histone methyltransferase that targets H3K4.[19] The effect of dCas9-or ZF-PRDM9 targeting to gene promoters on gene activation is moderate and much lower compared with dCas9- or ZF-VP64.[20] Interestingly, gene activation by ZF-PRDM9 is only persistent for nonhypermethylated genes.[20] This may be because DNA methylation excludes H3K4me3.

Targeted gene silencing by editing histone modification

The corresponding first-generation epigenetic editing tool for gene inactivation includes Kruppel-associated box (KRAB) and Sin3a, both of which recruit proteins that either catalyze repressive histone marks or remove active histone marks. KRAB is a protein domain conserved from yeast to human and is both necessary and sufficient to repress transcription in transgenic assays.[21] KRAB is targeted to promoters of genes for transcriptional repression.[22] KRAB recruits KAP-1, which serves as a scaffold to recruit SETDB1, a SET domain protein that catalyzes H3K9me,[23] and HDAC.

H3K9me is a marker for inactive promoters. H3K9me can also be catalyzed by SET domain−containing HMTs, G9a and SUV39H1. Targeting ZF-G9a or ZF-SUV39H1 to the promoters of endogenous genes is sufficient for gene repression and deposition of H3K9me, which spreads to 1 kb away from the ZF-binding site. The spreading of H3K9me is mediated by HP1. This suggests that H3K9me has a causative role in gene repression and is not merely a by-product.[24] Interestingly, recruiting HMT and HDAC at the same time can boost the repression effect,[24] which reflects natural KRAB function.

Like KRAB, Sin3a is a gene-silencing effector protein in epigenetic editing. Sin3a belongs to a transcriptional repression complex, which includes Sin3b, Mad1, and Max.[25] The Sin interaction domain (SID) of Mad1 has been fused with TALE for

targeted repression and shows better repression capability than TALE-KRAB.[26] Directly fusing Sin3a with TALE leads to the reduction of H3K9Ac, a histone marker of active transcription.[27] Sin3a is associated with HDAC PRD3 and is able to reduce level of H3K4me3, a histone mark for active promoters. The latter is potentially achieved by inhibiting S-adenosylmethionine (SAM), a donor for methyl groups for epigenetic and protein posttranslational modification.[28]

Target noncoding RNA

Similar to messenger RNA regulation, the programming of noncoding (nc) RNA includes regulation of its expression and RNA editing that rescues mutations. Besides manipulating DNA methylation and histone modification, one can use antisense oligonucleotide (ASO) to reduce ncRNA expression and virus delivery to increase ncRNA expression. The CRISPR system can also be used for targeted RNA editing, and it is also possible to regulate the spatial location of the long ncRNA using CRISPR-DISPLAY.[29]

Among the aforementioned methods to regulate ncRNA, ASO is the only approach that has been clinically approved. ASO is a chemically modified DNA molecule that binds to RNA target to form a DNA−RNA complex, which induces degradation of the target RNA. ASO can target short or long ncRNA. The main limitation of this technology, considering its clinical application, is that its effects are transient. This is because the ASO is cleaved once it binds to its target.

Developmental disease that have the potential to be treated with epigenetic editing

Epigenetic editing can potentially be applied to treat developmental diseases such as imprinting disorders and X-linked disorders (Fig. 13.1).

Imprinting disorders

Angelman syndrome

Etiology

Angelman syndrome is a rare neurodegenerative disease that causes mental retardation, epileptic seizures, and gait ataxia. The gene associated with this disease is *UBE3A* on the chromosome 15, an imprinted gene expressed on the maternal allele. UBE3A is an ubiquitin ligase, an enzyme that adds ubiquitin molecules to proteins to target them for degradation. UBE3A also functions as a transcription coactivator.[30] UBE3A localizes to both the nuclei and dendrites of neurons.[30] UBE3A targets P27, P53, EPHEXIN-5, and ARC for proteasome-mediated degradation. P27 and P53 are important for the proliferation of neurons.[31] EPHEXIN-5 is important for

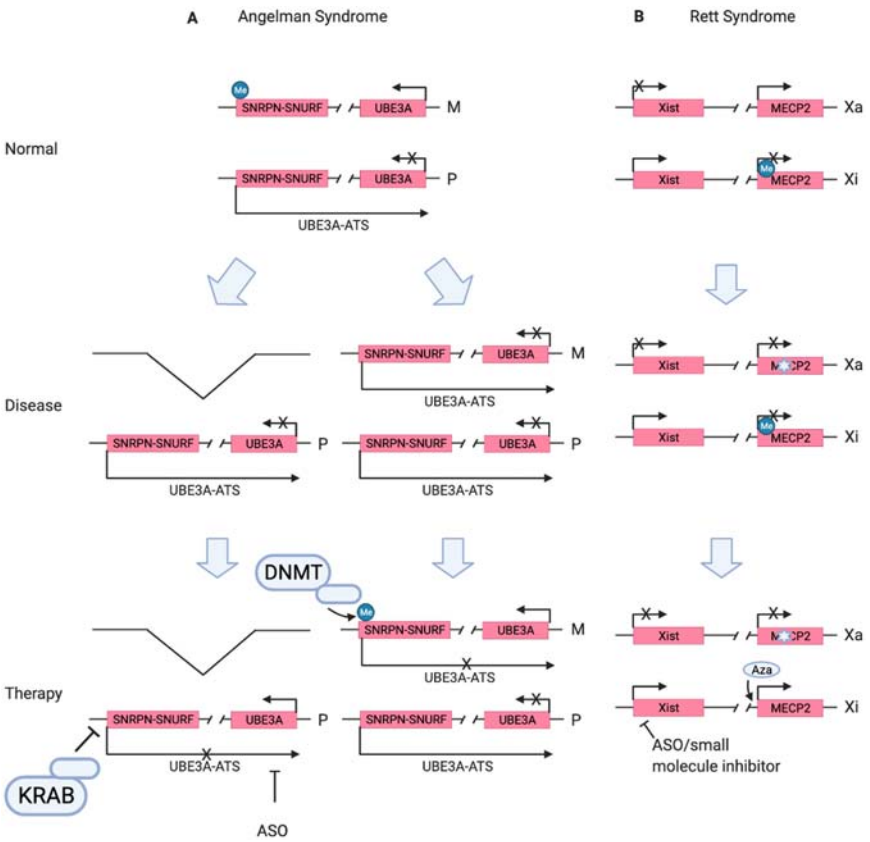

FIGURE 13.1 Summary of developmental diseases and epigenetic editing as a potential therapy.

(A) The Angelman syndrome caused by maternal deletion can be treated by reactivating the silenced paternal UBE3A by inhibiting the expression of paternal UBE3A-ATS through ASO or KRAB. The disease caused by maternal imprinting disorder can be rescued by restoring the methylation of the promoter of UBE3A-ATS through DNMT. M, maternal chromosome; P, paternal chromosome. UBE3A-ATS, antisense transcript of UBE3A. ASO, antisense oligonucleotide. KRAB and DNMT can be targeted to the loci through zinc finger (ZF) proteins or dCas9. (B) Rett syndrome is caused by heterozygous mutations in the *MECP2* gene, as illustrated by the star. Either using small molecule inhibitors to inhibit *XIST* transcription or the combination of ASO and Aza (DNMT inhibitor) to silence *XIST* and reactivate *MECP2* on the Xi is a potential therapeutic approach.

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synapse formation.[32] Elevated ARC increases α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor endocytosis and thus impairs synaptic transmission in excitatory neurons. In addition, as a transcriptional coactivator, UBE3A regulates the transcription of *CBLN1*[33] and *ARC*,[34] which are important in synaptic function. Thus, the neurons degenerate, and synapses are impaired in AS.

Normally, UBE3A antisense transcript (UBE3A-ATS) is expressed from the paternal allele as the promoter for maternal allele is hypermethylated. Interestingly, UBE3A only monoallelically expressed in neuron cells, because the UBE3A-ATS in nonneuron cells cannot elongate to *UBE3A* site and is subsequently terminated by a boundary enriched by multiple CCCTC-binding factor (CTCF) proteins.[35] Angelman syndrome is caused primarily by four genetic or epigenetic mechanism: (1) maternal deletion of a ~6 Mb region in 15q11.2−q13 encompassing the imprinting center and *UBE3A* gene, which accounts for more than 70% of patients and is phenotypically the most severe type; (2) mutation in the maternal copy of *UBE3A* accounts for 11% of patients[36]; (3) imprinting defects including deletion of the imprinting center, or the maternal methylation mosaicism of the exon 1 of *SNURF-SNRPN* (the promoter of *UBE3A-ATS*), which accounts for 6% of patients[37]; and (4) paternal uniparental disomy, which accounts for 3% of patients.[36]

Previous efforts to develop treatment

The concern of gene therapy for Angelman syndrome is that viral delivery of DNA or mRNA by virus can cause "gene overdose," and *UBE3A* duplication potentially contributes to autism.[38] As there is a silent allele of *UBE3A* on the paternal allele in Angelman syndrome patients, one attractive strategy is to reactivate this allele. This can be achieved by inhibiting the extension of the *UBE3A-ATS*. Drug screening using mouse cortical neurons found that topoisomerase I inhibitor significantly increased paternal *UBE3A* expression. However, this method lacks specificity since other genes can also be affected by topoisomerase I inhibitor. In addition, this drug has toxicity, making it difficult for clinical application.

Epigenetic editing to treat Angelman syndrome

Anti-UBE3A-ATS oligonucleotides can inhibit the extension of the *UBE3A-ATS* transcript in cultured mice neurons.[39] In cultured mice neurons, the ASO is tolerated and is effective in reducing *UBE3A-ATS* transcription and upregulating paternal *UBE3A* expression. When the ASO is administered once via intracerebroventricular injections, UBE3A expression in the cortex and spinal cord reaches 80% of the normal maternal allele for 4 months and then decreases to baseline. One month after the injection, performance in memory testing was improved. However, performance in marble burying, accelerating rotarod tests, and open field did not improve.

In a mouse neuroblastoma cell line, anchoring KRAB to the initiation site of the *UBE3A-ATS* reduced the transcription of *UBE3A-ATS*.[40] An HIV TAT domain for cell penetration was added to the ZF-KRAB construct. This allowed the fusion protein to cross the blood−brain barrier. The purified protein was delivered into the brain via peripheral injection, resulting in widespread distribution in the mouse brain. In the hippocampus and cerebellum, which contributes to the cognitive[41] and motor defects,[42] respectively, protein expression as measured by quantifying the immunohistochemistry signal in treated Angelman syndrome mice model achieved about 80% of the signal measured in wild-type control mice. One of the major limitations of this method is that the reactivation is transient, probably

because they injected purified protein. The half-life of ZF-KRAB is 8–24 h, so it requires injection every other day.

In addition, targeting dCas9-DNMT3A to exon 1 of *SNURF-SNRPN* in single oocyte can restore the DNA methylation on the TSS of UBE3A-ATS in oocytes, which can be maintained after in vitro fertilization.[43] Embryo transfer experiments and further behavior testing in mice confirmed that the somatic tissue of the offspring had established the methylation of *SNURF-SNRPN*. This type of epigenetic editing will be suitable for treating the 6% of Angelman syndrome patients with imprinting defects.

Prader–Willi syndrome

Etiology

Prader–Willi syndrome (PWS) is a disease manifested as overeating with psychotic and endocrine disorders. The imprinting center of PWS (PWS-IC) controls the paternal epigenotypes of genes including *MAGEL2*, *NDN*, and *SNURF-SNRPN* in 15q11.2-q13. These genes normally express their paternal copy. Research has highlighted that the C/D box small nucleolar (sno)RNA cluster *SNORD116* in the intron of *SNURF-SNRPN* is responsible for the majority of the PWS symptoms.[44] Deletion of *SNORD116* caused hypothalamus defects and hyperphagia, resulting in obesity in a subset of mice.[45] *SNORD116* is suggested to modulate many neuronal gene transcripts, including those that regulate the circadian clock and energy expenditure.[46] The majority of PWS patients have 5.4 Mb or 6 Mb deletion on the paternal 15q11.2-q13 that include the PWS-related genes mentioned before.[47] Approximately 20%–30% of the patients have maternal uniparental disomy. About 1%–3% PWS patients have imprinting disorders.[48]

Previous efforts to develop treatment

Growth hormone therapy can increase the height and reduce the body mass of the patients. However, whether the cognitive and behavior defects can be ameliorated by growth hormone treatment is still under investigation.

To relieve additional PWS defects, epigenetic editing to reactivate the maternal copy of *SNORD116* might be promising. The maternal allele of *SNORD116* is silenced by a complex formed by SETDB1 and the zinc finger protein ZNF274. ZNF274 is relatively specific for this locus since it has fewer than 500 binding sites in the genome. Moreover, ZNF274 recruits SETDB1 for gene-silencing effect. H3K9me3 mediated by histone H3K9 methyltransferase SETDB1 is suggested to be the mechanism for silencing *SNORD116*.[49] However, knocking down ZNF274 with RNAi resulted in only a moderate increase of maternal *SNORD116* expression, less than 1/1000 of the paternal copy.[49] This may be because the remaining ZNF274 can still recruit enough SETDB1 for repression, or alternatively because there are other proteins that recruit SETDB1 to the maternal *SNORD116* locus. Knocking down of SETDB1 by short hairpin (sh)RNA decreases the repressive histone mark at the maternal *SNORD116* and partially restores the maternal SNORD116 level.[49]

H3K9me2 mediated by histone H3K9 methyltransferase G9a is another potential mechanism for silencing *SNORD116*.[50,51] Small molecule compounds that inhibit G9a were screened based on reactivation of *SNURF-SNRPN* in mice embryonic fibroblasts.[52] The inhibitors bind and block the catalytic domain of G9a, and activated *SNORD116* in human skin fibroblast derived from a PWS patient. In a PWS mouse model, this inhibitor reactivated the maternal *SNURF-SNRPN* to 50% of the paternal level and rescued perinatal lethality model at the postnatal stage after a single intraperitoneal injection. This inhibitor was tolerated and acted through the reduction of H3K9me2 and H3K9me3.[52] Importantly, this method is not specific since the H3K9 methylation was reduced not only at the *SNORD116*.

Potential therapy by specific epigenetic editing

Potentially, fusing H3K9 demethylase LSD1 with dCas9 may specifically reduce repressive histone marks to allow *SNORD116* reactivation. Of note, the development of therapeutic methods needs to be evaluated on appropriate animal models. However, the effects of the treatment on obesity and hyperphagia cannot be assessed in current animal models.[47]

X-linked intellectual disability

Rett syndrome

Etiology

X chromosome inactivation (XCI) is a mechanism for dosage compensation between males and females in mammal, during which one of the two X chromosomes of females is transcriptionally silenced. The XCI is mediated by the expression of *XIST*, an lncRNA from the future inactivated X chromosome (Xi). The expression of *XIST* in early embryogenesis is sufficient and necessary for silencing of Xi. *XIST* represses transcription in *cis* only on the X chromosome from which it transcribed and is required for both establishment and maintenance of XCI.[53]

Rett syndrome (RTT) is a disease that causes the loss of motor and language ability, and it is mainly observed in females. RTT is caused by loss-of-function mutations on the X-linked gene *MECP2*. MECP2 is a master protein in transcriptional regulation in neurons and is important for synaptic connection.[54]

Previous efforts to develop treatment

Current treatment for RTT is mainly symptomatic. The directions for developing future therapy include targeting MECP2 or its downstream molecules. Because MECP2 is a master protein in transcriptional regulation, targeting any single molecule downstream of MECP2 can only ameliorate an aspect of the disease. Gene therapy has major problem with gene overdose, since *MECP2* duplication has been linked to autism spectrum disorders.

There is a wild-type *MECP2* allele in most female RTT patients. Thus, reactivating the wild-type *MECP2* in Xi might be a promising treatment for RTT.

Importantly, reactivation in the adult stage can rescue the phenotype. This is demonstrated by experiments where a stop cassette was inserted into the *MECP2* gene in male mice and deleted in adult stage.[55]

Developing specific epigenetic editing therapy

Small molecule inhibitors of *XIST* have been used.[53] shRNA screening was used to identify factors required for maintaining XCI in a female mouse embryonic fibroblast cell line. Inhibiting PDPK1, a serine−threonine kinase and regulator of phosphatidylinositol 3-kinase (PI3K)/AKT signaling, reversibly reactivates *MECP2* in mouse cortical neurons and fibroblast cells derived from RTT patients. However, this study did not follow-up with MECP2 protein detection or neuron function examination. In a later study by the same group, two drugs were used, an inhibitor of ACVR1, a molecule involved in cell signaling, and an inhibitor of SGK1, a PDPK1 effector substrates in mice and induced RTT neuron derived from human-induced pluripotent stem cell (iPSC).[56] In RTT neurons, *MECP2 mRNA* levels increased to about 10% compared with the positive control, and the neuronal morphology becomes comparable with the positive control. However, there is no quantitative mRNA measurement nor further functional examination in living mice in this study.

An ASO against XIST, and 5-Aza, a DNMT1 inhibitor, have been used in combination for therapeutic effects in RTT neurons in vitro.[57] 5-Aza can cross the blood−brain barrier, so the delivery is relatively easy. The XIST ASO and 5-Aza had a synergistic effect achieving 2.2% of reactivation compared with the *MECP2* mRNA level in the active allele. Although the reactivation seems to be moderate, the phenotypical improvement was significant, suggesting partial restoration of MECP2 expression is effective.[57] In the future, single-cell techniques could be applied to investigate whether the 2.2% overall reactivation is a huge reactivation from a few cells, or a moderate reactivation from a large population of cells.

However, the major limitation of these studies is specificity. Other genes subject to XCI have been reactivated even to a greater extent compared with *MECP2*. In addition, the efficacy of improving *MECP2* transcription is suboptimal. Considering the XCI is a complicated process involving different epigenetic players, it might be promising to combine different editing strategies, e.g., DNA methylation and histone modification on the *MECP2* locus to tackle RTT in the future.[7]

Fragile X syndrome

Etiology

Fragile X syndrome (FXS) is characterized by intellectual disability and an autistic spectrum phenotype. FXS is caused by loss of fragile X mental retardation protein (FMRP) encoded by the *FMR1* gene during brain development. FMRP is an RNA-binding protein in neurons. FMRP regulates membrane protein in the synapse and maintains normal synaptic plasticity. In FXS, a CGG trinucleotide repeat (>200 times) expansion mutation at the 5′ untranslated region (UTR) of *FMR1* is followed by DNA

hypermethylation, which results in heterochromatin formation at the *FMR1* promoter and subsequent silencing of *FMR1* expression in FXS. There is no cure for FXS so far.

Previous approach to develop treatment

There have been no effective treatment so far for FXS.[58] Since in FXS, the *FMR1* coding region is intact, epigenetically reactivating the *FMR1* gene is a promising approach. 5-azadC can irreversibly block DNMT. An initial test using 5-azadC is effective to reactivate mRNA but not translation,[59] since the multiple CGG repeats inhibit translation.[60] Combined treatment with 5-azadC and HDAC inhibitors causes a synergistic effect in reactivating *FMR1* transcription.[61] 5-azadC induces histone acetylation, and H3K4 methylation, and reduces H3K9 methylation at the *FMR1* locus.[62] However, the toxicity of 5-azadC has raised concern.[63] Moreover, the effects of 5-azadC treatment only last for 4 weeks.[64] This transient reactivation was resilenced by the recruitment of EZH2, a component of the polycomb repressive complex2 (PRC2) component, which catalyzes H3K27 trimethylation.[65] Compound 1a, an EZH2 inhibitor, was able to delay the resilencing, as the reactivation remains significant at 4 weeks posttreatment.[65] However, these previous efforts all utilized nonspecific inhibition of the epigenetic modifiers, which might lead to undesired side effects.[56]

Targeted epigenetic editing to treat FXS

Recently developed targeted DNA methylation editing tools[66] have been applied to treat FXS by demethylating the CGG repeats by dCas9-TET1. The heterochromatin status of the *FMR1* promoter was switched to an euchromatin state, as measured by anti-RNPII, H3K4me3, H3K27ac, HeK27me3, and H3K9me3 chromatin immunoprecipitation sequencing (ChIP-seq). The reactivation of *FMR1* can be observed in iPSC, iPSC-derived neurons (iN), and iN engrafted into the mouse brain. Finally, direct editing in postmitotic neurons in the mouse brain was able to reactivate *FMR1*, with FMR1 protein expression lasting for 1 month in the mouse brain.[67]

Insights for the application of epigenetic editing in the treatment of these developmental diseases

For any molecular therapy, there are several considerations including delivery, off-target effects, epigenetic efficacy, phenotypical potency, persistency, and immunogenic effects. Delivery and immunogenic effects are more general issues for gene editing, which have been reviewed in detail.[68–70] Thus, this chapter will cover off-target effects, efficacy of influencing transcription, phenotypical potency of the epigenetic editing, and persistency of the epigenetic modification (Fig. 13.2).

Off-target effects

Off-target is defined as the recruitment of DNA-binding protein–effector fusion to nontargeted sites in the genome.[71] We would like to use the DNA-binding protein

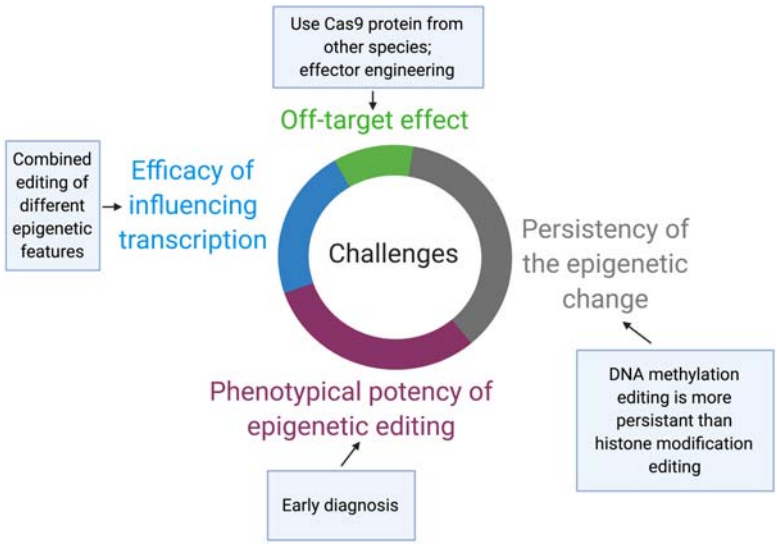

FIGURE 13.2 Summary of challenges of epigenetic editing for the treating developmental diseases.

This figure summarizes the challenges of epigenetic editing for treating developmental diseases and the potential solutions. The relative difficulty of each challenge is proportioned by the length of the colored band.

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dCas9 as an example to illustrate the off-target effect since it is most well characterized. One reason for off-target effect is that the choice of loci used for designing single guide (sg)RNA might be limited to the neighbor of the desired site. This limitation is due to the restriction by the protospacer-adjacent motif (PAM), a sequence required for target recognition by Cas9 (or dCas9). This restriction caused by PAM can be ameliorated by using dCas9 protein from another species with a different PAM sequence.[7] In addition, a PAMless editor, which is an engineered *Streptococcus pyogenes* Cas9 (spCas9), SpRY, recognizes NRN (R is A or G) and NYN (Y is C or T) PAMs instead of the NGG PAM for the original spCas9.[72] The design of this engineered variant was inspired by a high-throughput PAM determination assay that assesses the contribution of each amino acid residue in the PAM interacting domain of the SpCas9 protein.[72]

Another cause for an off-target is the effector fused with dCas9. This reason for off-target activity is specific to epigenetic editing. dCas9-DNMT3A without guide (g)RNA can cause global hypermethylation, potentially by the free diffusion of the catalytical domain of DNMT3A.[73] In an engineered cell line with DNMT3A–DNMT3L double knockout and DNMT1 knockdown, transfection with the catalytical domain of DNMT3A alone leads to 20% increase in global methylation.[73] One solution could be to mutate the amino acid in the catalytical domain of DNMT3A

that binds to DNA.[74] Such a mutation also led to a reduction of methyl transferase activity; however, using a Suntag system to increase the number of DNMT3A proteins recruited can compensate for the loss of efficacy.[74] For other effectors, another potential solution might be engineering the epigenetic effector for DNA binding—inducible activation.[7]

Efficacy of influencing transcription

How effectively can epigenetic editing change transcription in diseases depends on several aspects including the epigenetic mechanism of transcriptional regulation of the disease-related gene, and the delivery methods used. This section will focus on the epigenetic mechanism, rather than the delivery methods as they are not the focus of this chapter. Epigenetic editing could be effective when the epigenetic regulation of the disease-related gene was straightforward. For example, in Angelman syndrome, the goal of epigenetic editing was to reactivate the silenced paternal *UBE3A*. It was clear that the silence of paternal *UBE3A* is mediated by the UBE3A-ATS. Thus, using ASOs against UBE3A-ATS was able to reactivate the paternal UBE3A protein expression to 90% of the normal maternal copy.[39] However, when the epigenetic mechanism is more complicated, as with the X chromosome inactivation of *MECP2* locus in Rett syndrome, epigenetic editing using XIST ASO and DNMT inhibitor only increased *MECP2* transcription to 2% of that observed with the active X chromosome. The challenge here is that XCI involves multiple layers of epigenetic modifications, including DNA methylation, histone modification, and chromatin 3D structure. Editing a single epigenetic mark might not be efficient for transcriptional regulation. For example, depositing histone methylation, a repressive histone mark, was not able to repress gene transcription.[75] Thus, the solution could be to use a strategy of combined editing with multiple modifications achieved by using orthogonal Cas proteins such as Cas9 and Cpf1, from different species. In addition, multiple zinc finger proteins could be delivered to the same cell for combined epigenetic editing.

Phenotypical potency of the epigenetic editing

The developmental diseases discussed in this chapter are related to brain development, which occurs in the prenatal stage. Prenatal defects are usually diagnosed when the phenotypical defects are manifested, much later than when the defective molecular process happens. Thus, whether the disease is reversible at a later stage is an important question for treating developmental diseases.

For RTT, in the groundbreaking attempt in this direction in a mouse model, the *Mecp2* gene was silenced by inserting a stop cassette into the gene and then reactivating it through cassette deletion, thereby rescuing the RTT phenotype.[76] This proof-of-concept work gave rise the field of X chromosome reactivation (XCR). Similarly, for FXS, since excessive mGluR-mediated signaling due to lack of FMRP is critical for disease development,[77] administration of mGluR inhibitor

was effective in reversing the phenotype in young adult stage of FXS mice model, much later than the development of the disease.[78]

However, in AS, restoration of the paternal UBE3A in adult mice model by ASO targeting to UBE3A-ATS failed to rescue the majority of the neurocognitive defects of Angelman syndrome measured in behavior tests.[39] This was explained by later experiments that systematically examined the window of neurocognitive function impaired in Angelman syndrome. Although motor dysfunction can be rescued in adolescent, epilepsy, anxiety, and repetitive behavior can only be rescued in early development.[79] Thus, effective editing therapy should be combined with timely diagnosis.

Persistency of the epigenetic change

The persistency of the epigenetic change induced by the therapy is determined by two aspects, the epigenetic mechanism and the delivery method. Combining treatment with ZF-KRAB and ZF-DNMT3A-3L was able to achieve silencing that was resistant to transcriptional activators.[80] Similarly, recently published research shows that fusing KRAB and DNMT3A-3L to N and C terminal of dCas9 can achieve sustained epigenetic silencing cross-cell division and differentiation.[81] It is speculated that DNA methylation is the key for this persistency. In addition, certain histone modifications were also reported to be rigorous: HP1alpha can deposit H3K9me3 and induce the spread of heterochromatin state up to 10 kb, which can be inherited cross-cellular generations.[82] The basic speculation is that modified histone will recruit more histone modification enzymes so that this mark can be spread. However, this speculation has two additional requirements: the histone modification enzyme occasionally can lead to modification beyond nearby genes, and the cooperation between more than one edited nucleosome.[83]

The consideration of the delivery method is complicated. Since all the developmental diseases discussed in this chapter require brain delivery of the epigenetic editing machinery, crossing blood−brain barrier is required. For manipulation of DNA methylation or histone modification through DNA-binding protein−epigenetic effector fusion construct, purified protein with peptide that aids the crossing of blood−brain barrier was able to be delivered to the brain; however, its half-life is 8−24 h and therefore requires injection every the other day.[40] A single tail vein injection of AAV8 serotype that targets hepatocytes in the liver can lead to desired protein expression level for 6 months[84]; however, it is still difficult for AAV8 to cross the blood−brain barrier. Due to the transient nature of ASO-induced RNA degradation, this method of targeting noncoding RNA usually requires weekly injections.[85]

Future direction: manipulation of 3D chromatin topology to treat developmental diseases

Chromatin is packaged in nuclei as 3D topological structures. In megabase scale, the genome could be divided into A and B compartments based on high-throughput

chromosome conformation capture (Hi-C) data.[86] Each compartment is defined by the preference of intracompartment interaction over intercompartment interaction. Compartment A displaces the nucleus interior, with higher transcriptional activities. Compartment B occupies the nucleus periphery, with lower transcriptional activities.[86] In megabase and submegabase scales, the chromatin folds into topologically associated domains (TADs).[87] TADs are believed to increase the frequency of the interaction between the enhancer and promoter of genes within the domain, thus contributing to transcriptional regulation. TADs are likely composed of an insulated neighborhood, at the scale of about 200 kb, where enhancers prefer to interact with promoters by forming loops within the insulated region.[88] The formation of chromatin 3D structure is mediated by protein factors. TADs and the insulated neighborhood are mainly mediated by cohesin and CTCF binding at the boundary.[87] In vertebrates, enhancer–promoter loops can be mediated by YY1,[89] or site-specific factors such as Ldb1.[90]

To dissect the causal relationship between chromatin topology and transcription, specific manipulation of chromatin topology is required. Manipulation of insulated neighborhoods has been discussed in detail elsewhere,[88] and this section will focus on manipulation of chromatin loops, specifically. First, site-specific looping factors can be anchored to manipulate loops. Deng et al. have used a ZF protein to target Ldb1 to the hemoglobin locus.[90] Second, others have used nonmammalian factors to induce looping. Morgan et al. fused dCas9 with a chemically induced proximity system, e.g., the plant S-(+)-abscisic acid (ABA) signaling pathway.[91] dCas9 can also be fused with proteins from an optogenetic system that dimerize under blue light.[92] Lastly, endogenous chromatin organizer proteins such as YY1[89] and CTCF[93] have been fused to dCas9 to induce loops. Manipulation of enhancer–promoter loops was capable of regulating the transcription of some, but not all, genes tested.[90,94]

Interestingly, growing evidence supports the role of 3D chromatin topology in the developmental diseases discussed in the previous sections of this chapter. For example, reactivation of wild-type genes in the X chromosome might be useful for treating X-linked developmental diseases.[56] Several studies have suggested that chromatin topology might play a role in X chromosome inactivation. For instance, many TADs were attenuated across the inactivated X chromosome but were found to correlate with clusters of genes that had escaped XCI in the mouse brain.[95,96] Cohesin was found to interact with *XIST* and was depleted from the inactive X chromosome in mice.[97] Thus, in the future, manipulation of chromatin topology would be a new and exciting direction to treat diseases such as Rett syndrome. In addition, CTCF is involved in Angelman syndrome. In healthy individuals, at the Angelman syndrome loci, the paternal *UBE3A* is repressed by UBE3A-ATS in the neurons.[35] However, in nonneuronal cells, UBE3A is biallelically expressed, because UBE3A-ATS cannot elongate to the paternal *UBE3A* and thus is terminated by CTCF blocking.[35] CTCF occupies the site between the TSS of *UBE3A-ATS*, and the *UBE3A* gene restricts the long-distance interaction on each side of the CTCF-binding site[35] Thus, restoring CTCF binding locally might restore the restriction

and block the elongation of UBE3A-ATS in the paternal allele in neurons, thus reactivating the paternal *UBE3A* to potentially treat Angelman syndrome.

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