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Novel strategies to cure imprinting disorders

Abstract: In imprinting disorders, where the active copy of an imprinted gene is mutated or lost, there is a unique opportunity for causal treatment by unsilencing the other, dormant allele. Depending on the mechanism by which the allele is silenced, unsilencing can be achieved by epigenetic drugs, antisense-oligonucleotides (ASOs) or epigenome editing. While most of the research is still preclinical, first-in-humans studies with ASOs have started in 2020.

Keywords: imprinting disorders, activation of silent alleles, epigenetic drugs, antisense-oligonucleotides, epigenomic editing

Introduction

As in most genetic diseases, a causal therapy for imprinting disorders is not available. The clinical management of these patients is oriented towards ameliorating some of the clinical signs. It involves, for example, the administration of glucose in newborns with Beckwith–Wiedemann syndrome (BWS), insulin in infants with Transient Neonatal Diabetes Mellitus (TNDM), growth hormone in infants and children with Prader–Willi syndrome (PWS) or Silver–Russell syndrome (SRS) and GnRH analogs in children with Central Precocious Puberty 2 (CPPB2) (for the current status of the clinical management of imprinting disorders see Elbracht et al. in this issue). In some imprinting disorders such as BWS, TNDM and CPPB2 most of the clinical problems resolve over time and patients live a normal life. In PWS, Temple Syndrome, Schaaf–Yang syndrome, Angelman syndrome (AS) and Birk–Barel syndrome, the major clinical signs such as intellectual disability cannot be ameliorated. In Kagami–Ogata syndrome, many patients have thorax malformations and die very young.

What sets imprinting disorders aside from other genetic diseases is the fact that they involve genes which are expressed from one allele only (either the maternal or the paternal allele; see Prawitt and Haaf in this issue). The disease results from the loss of the one and only active gene (this is most often the case) or from a double dose of an active allele (which is less often the case). This feature allows for a novel therapeutic strategy which is not available in other genetic diseases (see below). Since imprinted genes are likely to occur in gene clusters, it is not always clear if only one or which of the genes in the affected gene cluster contributes to the disease. This makes the development of causal therapies in some disorders difficult.

As discussed above, an overarching and life-long problem in several imprinting disorders is intellectual disability, which occurs to a different degree in the different disorders, but also to a different degree in different patients with the same disorder. Of note, in none of these disorders, intellectual disability is progressive or associated with neurodegenerative disease. This gives hope that intellectual disability may in principle be ameliorated. In fact, studies in mice with AS have shown that some features of the disease are partially reversible upon switching on the relevant gene after birth [1]. Although the brain is plastic, plasticity is gradually lost upon aging. This calls for an early intervention. Brain neurons, however, are probably the most difficult target for causal therapies: the blood–brain barrier makes it necessary to use small molecules that can pass this barrier, or to use intrathecal or intracerebral injections for drug delivery.

Probably, only imprinting disorders with a life-long handicap are candidates for the development of a genetic therapy. As in other diseases, there must be a reasonable chance that the clinical features can be ameliorated, and the benefits must outweigh the side effects. One option for treating severe imprinting disorders affecting the brain is gene replacement therapy with adeno-associated viruses, which can deliver genes to the central nervous system (CNS). Gene replacement therapy is not discussed any further in this article, because most of the problems and chances associated with this type of therapy are not specific to imprinting diseases. There is, however, a potential problem with gene replacement therapy in imprinting disorders. Imprinted genes are dosage-sensitive. This is probably one reason why these genes have become subject to genomic imprinting and mono-allelic expression. In gene replacement therapy there is a risk that the delivered gene copy is expressed at a much higher level compared to the

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one active allele in a normal cell. Overexpression of an imprinted gene may cause harm. Therefore it would be of utmost importance to tightly control the level of expression of the introduced gene copy, which is very difficult to do. Nevertheless, some researchers are exploring this possibility in AS.

Activation of the silent allele

In imprinting diseases, where the one and only active copy of an imprinted gene is lost, there is a unique opportunity for causal treatment. In all of these diseases, and independently of the molecular defect (chromosomal deletion, uniparental disomy, imprinting defect or gene mutation), there is at least one intact, but silent gene copy on the other parental chromosome. Unsilencing of this gene copy would restore gene activity, which may ameliorate some of the clinical signs. Since unsilencing of a gene is likely to be inefficient, there is probably no risk of overexpressing the gene in the case of uniparental disomy or imprinting defects, where two inactive copies would be unsilenced upon treatment. Similar to gene replacement therapy and other forms of therapy, however, any drug used for unsilencing a gene in the brain must be able to cross the blood–brain barrier or, if this is not the case, be injected into the CNS.

For unsilencing the inactive copy of an imprinted gene, the silencing mechanism must be understood in detail. There are several ways by which imprinted genes are silenced: directly by DNA methylation of the promoter or indirectly by enhancer blocking or transcriptional interference. Histone modifications also play a role. It is probably easier to interfere with indirect silencing mechanisms, which are somewhat leaky, than to reverse methylation-mediated promoter inactivation.

In principle, two strategies can be envisaged: the use of "epigenetic drugs," which have a general effect on DNA methylation, histone modification or long noncoding transcripts, or a locus-specific approach. Examples for the first strategy are the use of azacytidine to inhibit DNA methylation, the use of valproic acid to inhibit histone deacetylation and the use of topotecan to suppress very long non-coding RNAs. These compounds can be administered very easily, but most of them cause side effects. While valproic acid is a relatively safe drug used to treat epilepsy, azacytidine and topotecan have severe side effects, which are acceptable only in life-threatening conditions such as cancer. In contrast, a locus-specific approach is likely to have fewer side effects, but is more difficult to

establish. Here we discuss two candidate imprinting disorders which may in the future be treated by activating the normally silent allele: AS and Birk–Barel syndrome.

Angelman syndrome

AS is caused by the loss of function of the maternal *UBE3A* allele [2]. In contrast to most other imprinted genes, monoallelic expression of *UBE3A* is cell type-specific and restricted to brain neurons. Silencing of the paternal *UBE3A* allele in these cells does not occur by DNA methylation, but by transcriptional interference with a long non-coding RNA (*SNHG14*), which starts at the *SNRPN* or *SNRPN* upstream promoters [3, 4] (Fig. 1). *SNRPN* is located ~500 kb centromeric to *UBE3A*. The two genes are in a convergent orientation and transcribed from opposite strands. The *SNRPN* promoter/exon 1 region carries the primary genomic imprint and is methylated on the maternal chromosome. As a result, *SNRPN* and *SNHG14* are expressed only from the paternal allele. *SNHG14* serves as a host for ~80 small nucleolar RNAs (snoRNAs). The loss of the *SNORD116* gene cluster appears to play a major role in PWS. In brain neurons, and only in these cells, *SNHG14* transcription extends beyond the *SNORD* genes – and in antisense orientation – into the *UBE3A* gene. Convergent transcription of the two genes results in RNA polymerase collision, which occurs predominantly in intron 4 of *UBE3A* [5]. As a result, no full-length *UBE3A* RNA and hence no UBE3A protein is produced from the paternal allele.
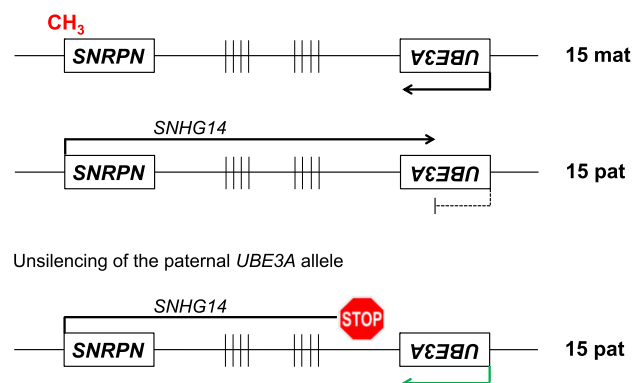

Figure 1: The Angelman syndrome locus on chromosome 15. The *SNRPN* and *UBE3A* genes are shown as boxes and *SNORD* genes as vertical lines (not drawn to scale). Arrows indicate gene expression. The paternal *UBE3A* allele can be unsilenced by preventing *SNHG14* transcription through the *UBE3A* gene with topoisomerase inhibitors, ASOs or CRISPR/Cas.

Patients with a maternal deletion 15q11q13 or a maternal *UBE3A* mutation have one intact paternal copy, which is silenced in the brain; patients with uniparental paternal disomy or an imprinting defect have two such copies. Therefore, gene replacement therapy is unnecessary if the silent paternal *UBE3A* allele can be activated. Since this allele is silenced by *SNHG14*, preventing *SNHG14* transcription through the *UBE3A* locus might result in the production of full-length paternal *UBE3A* transcripts (Fig. 1, lower part). A proof-of-principle for this approach was obtained in AS mouse models. Huang et al. showed that topoisomerase inhibitors such as topotecan reduce *Snhg14* expression and thus unsilence the dormant paternal allele of *Ube3a* in neurons [6]. Meng et al. introduced a poly(A) stop cassette into the paternal chromosome between the *Snord* gene clusters and the *Ube3a* gene [5]. Truncation of *Snhg14* leads to activation of the paternal *Ube3a* allele and ameliorates several disease-related symptoms, including motor coordination defects, cognitive deficits and impaired long-term potentiation. Since topoisomerase inhibitors affect the expression of many long non-coding RNAs [7] and genetic engineering in the brain of patients is not yet feasible (although research in this direction is underway [8]), other routes of unsilencing the paternal *UBE3A* allele need to be explored.

As shown by Meng et al. in an AS mouse model [9], such a route could be the use of antisense oligonucleotides (ASOs), which typically are ~20 nucleotides long. ASOs directed against *Snhg14* between the *Snord* genes and *Ube3a* reduced the expression level of the *Ube3a*-antisense part of *Snhg14* (without affecting the expression of *Snrpn* and the *Snord* genes), sustained unsilencing of paternal *Ube3a* in neurons *in vitro* and *in vivo* and ameliorated some cognitive deficits. Since ASOs cannot cross the blood–brain barrier, they have to be administered by intrathecal injection. As shown by the successful ASO treatment of spinal muscular atrophy, there are very few side effects. Furthermore, ASOs appear to be rather stable so that injections are needed only every four months. In fact, therapeutic ASOs are coming of age [10]. Three pharmaceutical companies plan to start or have already started the first clinical trials in humans with ASOs. The primary goal of these studies is to assess the safety of ASO treatment.

Birk–Barel syndrome

Birk–Barel syndrome is caused by mutations of the maternal *KCNK9* allele [11]. *KCNK9* is expressed in the majority of neurons throughout the CNS [12, 13]. Parent-specific differential DNA methylation at a CpG island overlapping the paternally expressed human *PEG13* non-coding RNA gene is assumed to be involved in silencing of the paternal *KCNK9* allele [14]. *PEG13* is located ~400 kb telomeric to *KCNK9*. The *PEG13* differentially methylated region is methylated on the maternal allele, binds CTCF-cohesin on the unmethylated paternal allele and has enhancer-blocking activity [15]. A methylation-sensitive enhancer-blocking mechanism involving CTCF–cohesin-mediated chromatin looping is well known to regulate reciprocal *IGF2*/*H19* imprinting [16]. Similarly, mutually exclusive CTCF–cohesin-mediated chromatin looping between a brain-specific enhancer region, enriched for histone H3 lysine 27 acetylation (H3K27ac) and histone H3 lysine 4 monomethylation (H3K4me1), and either the *PEG13* or the *KCNK9* promoter is proposed to control brain-specific reciprocal mono-allelic expression of the two genes [15] (Fig. 2).

Patients with a maternal *KCNK9* mutation have a paternal gene copy which is epigenetically silenced yet struc-
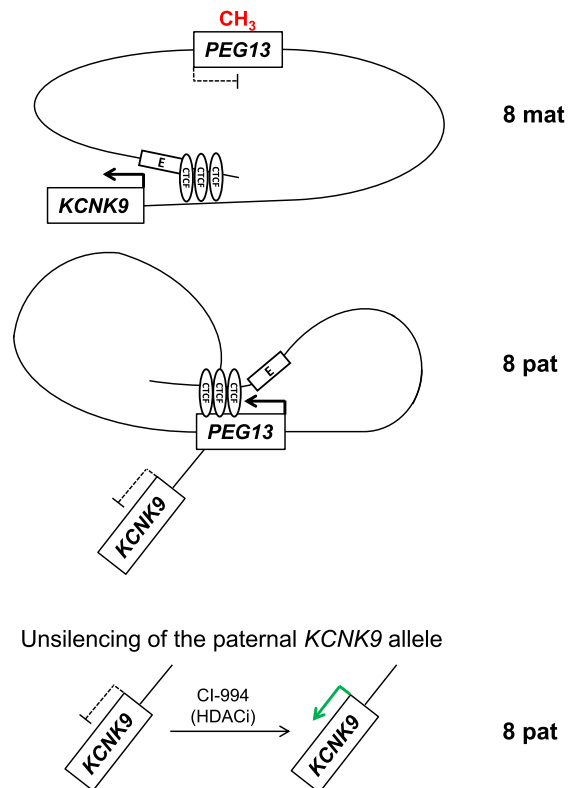

Figure 2: The Birk–Barel syndrome locus on chromosome 8. The *KCNK9* and *PEG13* genes are shown as boxes, the brain-specific enhancer as a small box and the CTCF molecules as ellipsoids (not drawn to scale). Arrows indicate gene expression. The paternal *KCNK9* allele may be unsilenced through increasing H3K27ac using the HDAC inhibitor (HDACi) CI-994.

turally unimpaired. A recent study in a mouse model for Birk–Barel syndrome showed partial behavioral rescue of mice with heterozygous deletion of the active maternal *Kcnk9* allele (*Kcnk9*KO^mat) compared to *Kcnk9* full knock-out animals [17]. This partial rescue was attributed to a brain region-specific leakiness of the *Kcnk9* imprint leading to upregulation of the repressed paternal allele in the case of loss of the maternal allele. These findings prompted the authors to explore possibilities to further de-repress the paternal allele in *Kcnk9*KO^mat mice using epigenetic modulators. Administration of the second-generation histone deacetylase (HDAC) inhibitor CI-994 by intraperitoneal injection further activated expression from the paternally silenced *Kcnk9* allele in several brain regions and fully rescued the behavioral phenotype. Interestingly, CI-994-mediated activation of the paternal *Kcnk9* allele did not involve DNA methylation changes at the *Peg13* DMR, but an increase of H3K27ac at regulatory elements in the *Kcnk9* promoter and intronic regions, particularly at the paternal allele [17].

Epigenetic modulators are often criticized to be less selective and bind many unspecific targets in the genome. They may cause various side effects, like tumor formation as well as mental and metabolic disorders. Though, valproic acid is a first-generation HDAC inhibitor widely used for the therapy of seizures and bipolar disorder for which even anticancer effects have been repeatedly reported [18, 19]. A beneficial role of the second-generation, specific class I HDAC inhibitor CI-994 is also indicated based on its documented inhibitory effect on epithelial-mesenchymal transition and neuroprotective properties in the CNS [20, 21]. In addition, no adverse effects of CI-994 treatment were observed in several mouse behavioral paradigms [17, 22]. Furthermore, the expression of other nearby imprinted genes within the imprinted cluster on mouse chromosome 15 was not altered by CI-994 treatment of *Kcnk9*KO^mat mice [17]. Thus, a rather specific effect of CI-994 in *Kcnk9*KO^mat animals may be assumed, which could make its use in patients with Birk–Barel syndrome and possibly also other imprinting disorders safe and feasible.

Imprint editing

Since the primary genomic imprint is DNA methylation, site-specific methylation or demethylation should make it possible to activate silent alleles or to correct imprinting defects. The challenge is to direct DNA methyltransferases or methylcytosine dioxygenases, which initiate

DNA demethylation, to the target sites. The DNA modifying enzymes cannot recognize DNA sequence context (apart from CpG) and need the help of guidance molecules. *In vivo*, transcription factors and histone modifications serve this role. Over the recent years, three types of DNA binding molecules capable of recognizing specific DNA sequences have been used in experiments: zinc finger proteins, transcription activator-like effectors and the guide RNA (gRNA)/Cas9 system [23]. The gRNA/Cas9 system has turned out to be the most versatile system for epigenome editing [24, 25]. In this system, a dead Cas9 enzyme (dCas9), which has no nuclease activity, is fused to the catalytic domain of DNMT3A to induce DNA methylation or the catalytic domain of TET1 to induce DNA demethylation. Short gRNAs direct the engineered enzymes to the target site (Fig. 3). Liu et al. (2018) reported demethylation of the hypermethylated CGG repeat expansion at the *FMR1* locus in induced pluripotent stem cells and derived neurons after infection with lentiviruses expressing dCas9-Tet1 and a single guide RNA [26].
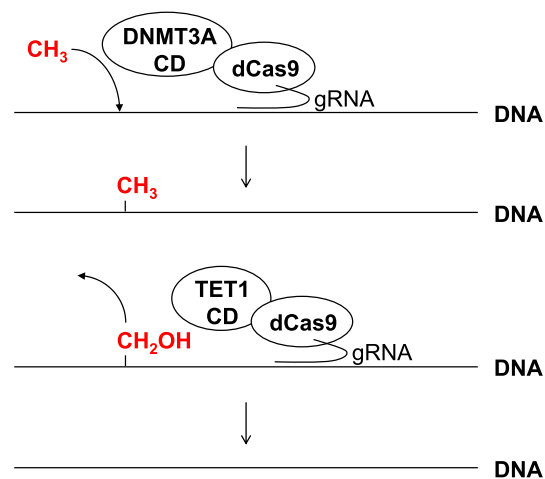

Figure 3: Epigenome editing. With the help of a locus-specific guide RNA, a dead Cas9 enzyme fused to the catalytic domain (CD) of a DNA methyltransferase (DNMT3A) or the methylcytosine dioxygenase 1 (TET1) is guided to the locus of interest to methylate or demethylate it.

In principle, it should be possible to use epigenome editing tools to edit genomic imprints, but this needs to be shown. Even if it works in cell culture, it is unclear whether this approach can eventually be used to treat patients with an imprinting disorder. There are several problems that need to be considered: 1. The constructs are very big and have to be delivered to the target cells by viral vectors. 2. Although the gRNA/Cas9 system is very specific, it is well

known that off-target effects can occur. Nevertheless, imprint editing remains a fascinating possibility.

Summary and outlook

Although imprinting diseases are rare and a causal therapy may be possible in only a few of them, researchers and pharmaceutical companies are exploring novel therapeutic strategies such as those described here. This endeavor is mainly driven by scientific interests, but also by the lobbying of parents' support groups. While most of this research is at the proof-of-principle or preclinical stage, first-in-humans studies with Angelman-ASOs have started in 2020. If these studies are successful, others may follow.

Compliance with ethical guidelines: For this article, the authors did not conduct any studies in humans or animals. For the studies listed, the ethical guidelines stated there apply.

Conflict of interest: B. Horsthemke is a consultant for IONIS Pharmaceuticals. He has received fees and travel grants from IONIS Pharmaceuticals and Pfizer. He holds two patents with Roche. U. Zechner declares no competing interests.

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