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Epigenetic therapy of Prader–Willi syndrome

YUNA KIM, SUNG EUN WANG, and YONG-HUI JIANG

Department of Pediatrics, Duke University of School of Medicine, Durham, North Carolina; Department of Neurobiology, Duke University of School of Medicine, Durham, North Carolina; Department of Program in Genetics and Genomics, Duke University of School of Medicine, Durham, North Carolina; Department of Program in Cellular and Molecular Biology, Duke University of School of Medicine, Durham, North Carolina.

Abstract

Prader–Willi syndrome (PWS) is a complex and multisystem neurobehavioral disorder. The molecular mechanism of PWS is deficiency of paternally expressed genes from the chromosome 15q11-q13. Due to imprinted gene regulation, the same genes in the maternal chromosome 15q11-q13 are structurally intact but transcriptionally repressed by an epigenetic mechanism. The unique molecular defect underlying PWS renders an exciting opportunity to explore epigenetic-based therapy to reactivate the expression of repressed PWS genes from the maternal chromosome. Inactivation of H3K9m3 methyltransferase SETDB1 and zinc finger protein ZNF274 results in reactivation of *SNRPN* and *SNORD116* cluster from the maternal chromosomes in PWS patient iPSCs and iPSC-derived neurons, respectively. High content screening of small molecule libraries using cells derived from transgenic mice carrying the *SNRPN*-GFP fusion protein has discovered that inhibitors of EHMT2/G9a, a histone 3 lysine 9 methyltransferase, are capable of reactivating expression of paternally expressed *SNRPN* and *SNORD116* from the maternal chromosome, both in cultured PWS patient-derived fibroblasts and in PWS mouse models. Treatment with an EHMT2/G9a inhibitor also rescues perinatal lethality and failure to thrive phenotypes in a PWS mouse model. These findings present the first evidence to support a proof-of-principle for epigenetic-based therapy for the PWS in humans.

INTRODUCTION

Prader–Willi syndrome (PWS) is a complex and multisystem neurobehavioral disorder, first described by Prader et al in 1956 based on its characteristic clinical features. These clinical features have since been well-delineated through natural history studies.^{1–3} The exact incidence of PWS remains unknown, but is estimated to be around 1 in 15,000–20,000 live births.¹

Most PWS cases are sporadic, but a small number of familial cases have been reported. In the 1980s, high-resolution chromosome analysis led to the discovery that PWS patients have a chromosomal deletion of 15q11-q13.^{4,5} This same deletion is also implicated in Angelman syndrome (AS), a severe neurodevelopmental disorder characterized by profound intellectual

disability and epilepsy.⁶ PWS and AS have since become prototypes for genomic imprinting disorders in humans.^{7,8} In both disorders, the deletion is associated with a different parental origin.^{9,10} The PWS deletion is of paternal origin, while in AS the same deletion is of maternal origin. Studies of PWS patients over the past 3 decades, and in particular, of rare cases with atypical etiologies, have exponentially expanded our understanding of the disorder on a molecular level.¹¹ A large number of studies investigating genomic imprinting mechanisms in mammals target the 15q11-q13 chromosomal region in humans and its homologous region in the mouse central chromosome, 7C.^{7,8} Despite substantial progress, the exact molecular pathogenesis of PWS has not been elucidated completely. This knowledge gap has limited the development of treatments that target its underlying genetic defects. Here, we review the major advances in the molecular study of PWS and discuss current and future perspectives on the development of epigenetic-based molecular therapies.

THE NATURAL HISTORY OF PWS

The major clinical manifestations of PWS are specific to the developmental stage of the patient (Table I).^{1,12,13} Clinical presentations likely begin in the prenatal stage, but there are few documented reports of abnormal findings during this period.¹³ In newborns, hypotonia and feeding difficulties are immediately noticeable, but gradually improve over the first 2 years of life. In most cases, interventions such as feeding assistance are necessary to maintain normal growth. There is a short period in middle infancy (2–4 years of age) in which feeding and growth appear relatively normal.¹⁴ During later infancy or early childhood, excessive feeding, or hyperphagia, becomes a significant problem. In most cases, this will develop into morbid obesity without clinical intervention.^{3,15,16} Motor milestones and language development are typically delayed, but usually only to a mild or moderate degree.^{2,17} Mild to moderate cognitive impairment is also common.¹⁸ Behavioral problems with obsessive-compulsive characteristics are frequently observed,^{2,19} and these individuals have a high risk of developing psychosis as adults.²⁰ Hypogonadism is present in both males and females, and manifests as genital hypoplasia, incomplete pubertal development, and infertility. Short stature is common and often presents with facial features characteristic of PWS, small hands and feet, strabismus, and scoliosis.¹

Molecular bases of PWS.

Historically, PWS is considered as a contiguous chromosomal microdeletion syndrome and a prototype for “genomic disorder.”²¹ In 1980, Ledbetter et al first reported chromosome 15q11-q13 deletion in patients with PWS using the then newly developed high-resolution chromosome banding technique.⁵ In 1989, Nicholls et al identified maternal uniparental disomy (UPD) 15. This discovery provided the first evidence of genomic imprinting in humans, and indicated that the molecular progression of PWS is impacted by the parent of origin, which was termed the “parental origin effect.”¹⁰ The majority of PWS patients have a large deletion (~6 Mb) of the paternal chromosome 15q11-q13. A significant subset of cases have maternal UPD 15. In rare cases of PWS, there is a separate class of imprinting defect in which there are no large chromosomal deletions or maternal UPD 15; instead, the maternal epigenotype is characterized by DNA methylation (5mC) at the 5' CpG island of the *SNRPN* gene in the 15q11-q13 region.²² The small deletions upstream of the *SNRPN* gene within

the 15q11-q13 identified in rare PWS patients delineate a critical regulatory element designated as imprinting center (IC).^{23,24} In a recent report, Butler et al summarizes the genetic findings from 510 individuals with PWS (Fig 1).²⁵ Of these, 60% have a ~6 Mb 15q11-q13 deletion in the paternal chromosome; 36% have a maternal UPD 15; and 4% have imprinting defects. The report found that 60% of cases have paternal deletions, which is lower than the historically reported ~70% of PWS cases,¹² and suggesting that the latter may be an overestimate due to the smaller sample size. Among the 303 cases with deletions, 38.9% have a 15q11-q13 type I deletion (deletion between breakpoint I and III, Fig 2), 165 (54.5%) have a 15q11-q13 type II deletion (deletion between breakpoints II and III), and 6.6% have a larger (>6 Mb) or in rare cases with a smaller (<1 Mb) atypical 15q11-q13 deletion. For the 185 cases with maternal UPD 15, 29.8% have maternal heterodisomy 15, 57.7% have segmental isodisomy 15, and 12.5% had total isodisomy 15. The average size of segmental isodisomy 15 was about 25.1 Mb with a range of 5–67.4 Mb, commonly in the 15q11-q13 and 15q26 regions.

Genotype and phenotype correlations for PWS.

There is no phenotypic feature that is known to correlate exclusively with any 1 of the 3 main molecular mechanisms (paternal deletion, maternal UPD 15, and imprinting defect) that result in PWS.¹² However, the differences in the frequency or severity of certain features are reported among different genotypes, primarily between the large paternal deletion and maternal UPD 15 for which a large number of cases are available for study. The molecular characteristics of different classes may predict that the individuals with imprinting mutation are more similar to the group with the maternal UPD 15. Individuals with a paternal deletion show more prominent feeding problem sleep disturbance, and speech articulation impairment.²⁶ However, the difference between type 1 and type 2 deletion appears not major and also inconsistent among different studies.^{2,19,27,28} Individuals with maternal UPD 15 have a slightly higher verbal IQ and milder behavior problems^{19,29} but are more likely to have psychosis in adult^{30,31} and autism spectrum disorder.^{32,33} In one study, as many as 62% of individuals with maternal UPD 15 develop atypical psychosis compared with 16% of those with a paternal deletion.³⁴ These observations provide initial clues for further investigation in a larger cohort and with a better phenotyping tool are warranted.

The imprinted domain, IC, and paternally expressed genes.

Discovery of the paternal origin effect in PWS indicates that the candidate gene or genes causing the disorder must undergo genomic imprinting that is either exclusively or preferentially expressed in the paternal chromosome.^{8,35} Indeed, over a dozen genes have been identified that are exclusively expressed in the paternal chromosome ubiquitously or in tissue specific manner within a ~3 MB interval from the breakpoint 1 (BP1) or breakpoint 2 (BP2) to *UBE3A* (Fig 2). Several genes known to be bi-allelic based on tissue sample analyses are also mapped in this interval. A bipartite IC that regulates in cis imprint resetting and imprint maintenance in the entire 15q11-q13 imprinted domain is mapped to upstream of *SNRUF/SNRPN* gene.^{23,24} All reported IC deletions include promoter/exon 1 of a paternally expressed *SNRUF/SNRPN* gene and overlap a well-defined CpG island in this region. The smallest IC deletion identified from a patient is ~4 kb and spans the promoter/exon 1 of the *SNRPN* gene.^{11,36} How the IC regulates the expression of imprinted genes

across the domain has not been fully elucidated.^{11,37-39} The PWS—IC is differentially regulated by several epigenetic modifications, including DNA methylation, histone methylation, and acetylation.^{23,40,41} Differential DNA methylation (5mC) is the best-recognized modification for the reference of parental identity.²³ In the literature, the CpG island of *SNPRN* within the PWS—IC region is the most reported region for DNA methylation. The PWS—IC is unmethylated on the paternal chromosome but methylated on the maternal chromosome by 5mC. DNA methyltransferases (DNMT1 and DNMT3A/B) play a role in both the establishment and maintenance of DNA methylation.^{39,42,43} Deficiency of DNMTs in mouse models results either embryonic or early postnatal lethality.^{44,45} Deficiency of *Dnmt1* in mice leads to loss of the methylation in PWS—IC and the bi-allelic expression of paternally expressed *Snrpn*.⁴⁵ The paternal genome undergoes active demethylation, in part through action of TET3, a ten-eleven translocation gene family member, which converts 5-methylcytosine to 5-hydroxymethylcytosine.⁴⁶ The maternal genome undergoes passive demethylation through multiple cell divisions. Differential DNA methylation that is placed on the PWS—IC in the germline is maintained following fertilization, despite the extensive reprogramming that occurs as the genomes are prepared for embryonic development. Interestingly, a recent genome wide high-resolution methylome analysis has revealed a more complex parent-of-origin specific differential methylated regions (DMRs) in the 15q11-13 PWS/AS imprinting domain.⁴⁷ In contrast to the pattern in PWS—IC, the entire region of 15q11-q13 displays an almost continuous preferential paternal methylation but interposed “spikes” of maternal methylation. The maternally methylated DMRs are mostly located at regulatory features (promoters, promoter flanks, and transcription start sites), whereas the paternally methylated DMRs are more evenly spread with repeats and heterochromatin in the region. The relevance of this pattern of DMRs to the imprinted expression of genes in the region remains unclear and is warranted for further investigation.

The critical region and candidate genes for PWS.

More than a dozen of paternally expressed coding genes, *SnoRNA* clusters, and long noncoding RNA transcripts have been identified from the PWS candidate region (Fig 2). Conceptually, all of them are candidate for PWS because the expression of these genes is absent in PWS patients with a large deletion, maternal UPD, and IC defect. *MKRN3*, *NDN*, *MAGEL2*, *NPAP1*, and *SNURF-SNRPN* are 5 protein coding genes. The specific functions for each gene and their relevance to the PWS phenotypes remain obscure. Mutant mice for *NDN*, *MAGEL2*, and *SNURF-SNRPN* genes have been generated and characterized (see more discussion in section of PWS models below). *MKRN3* is believed to play an important role in regulating the hormonal axis related to the puberty development in human because loss of function mutations in *MKRN3* is found in several families with an idiopathic precocious puberty.⁴⁸ There are 6 clusters of *SnoRNA* including *SNORD64*, *SNORD107*, *SNORD109A/B*, *SNORD116*, and *SNORD115* in the region between *SNRPNR* and *UBE3A*. They belong to the class of C/D box *SnoRNAs*.⁴⁹ *SNORD115* is implicated in the RNA editing of *5HTR2C* receptor but function for other *SnoRNA* clusters remain poorly understood.^{50,51} Similarly, little is known for the function of the majority long noncoding RNA transcripts in the region. Some of them are known to serve as the host transcripts for *SnoRNA* clusters. Two newly characterized *SPA1* and *SPA2* transcript, a long

noncoding RNA that is 5' *SnoRNA* capped and 3' polyadenylated are implicated in the RNA protein binding and alternative splicing of mRNA.⁵²⁻⁵⁴

Over the last decade, the application of new genomic technologies for molecular diagnostics, such as high-resolution copy number variant analyses by array Comparative Genomic Hybridization and whole exome sequencing, has revealed rare and subtle genetic defects in the chromosome 15q11-q13 in the individuals with a clinical or suspected diagnosis of PWS. In 2008, a case study of a clinically diagnosed PWS patient identified a small ~400 kb microdeletion spanning the *SNORD116* cluster⁵⁵ (Fig 2). The deleted interval also contains the host noncoding RNA transcripts that overlap with *IPW* transcripts, and a newly characterized *SPA1* and *SPA2* transcript.^{52,53} Similar findings are also reported in subsequent cases.⁵⁶⁻⁵⁸ The smallest microdeletion is restricted to the *SNORD116* cluster and its host noncoding RNA transcript, a portion of *IPW*, and *SPA2* transcript based on the genome annotation.⁵⁶ Molecular findings from these rare cases together indicate that a region between *SNRPN* and *UBE3A*, the *SNORD116* cluster in particular, may be critical to the key clinical presentations of PWS. However, it does not exclude a possibility that disruption of *SPA2* and *IPW* as well as the host transcript for *SNORD116* may also play a role because other studies have suggested that the noncoding RNAs of *SPA2* and *IPW* may have functions in regulating other genes.^{52,59} Over the past 5 years, whole exome sequencing has been used routinely to evaluate a small number of patients who have clinical features overlapping those of PWS or, in some cases, who were referred for the evaluation of autism spectrum disorder and intellectual disability. These clinical studies have revealed pathogenic single nucleotide variants in the paternal allele of the *MAGEL2* gene.^{60,61} These cases were later named Schaaf—Yang syndrome⁶² because of their distinct or otherwise consistent clinical features, such as autism spectrum disorder and joint contractures, which are not typically seen in PWS. Intriguingly, protein truncating mutations of *MAGEL2* are also associated with several cases who have a primary diagnosis of either arthrogryposis multiplex congenita or Chitayat—Hall syndrome, and whose features do not significantly overlap with those of PWS or Schaaf—Yang syndrome.^{63,64} The molecular basis of the pleiotropic presentation associated with *MAGEL2* mutations is not well understood. PWS patients with paternal deletion, maternal UPD 15, and IC defect do not express *MAGEL2*, and this deficiency would be expected to contribute to some of their clinical presentations. However, if *MAGEL2* mutations actually result in stable truncated proteins, and therefore act as a gain of function or dominate negative mutation mechanism, interpretation of truncating mutations of *MAGEL2* would be different. Further investigation is certainly warranted to test various hypotheses related to the role of *MAGEL2* in these different clinical presentations.

Current clinical treatments for PWS.

Like most human genetic diseases, there are no clinical targeted molecular therapies for PWS patients. Current management is symptom-based or recreational therapy for medical or behavioral problems.^{1,65} Special feeding techniques, including specialized nipples or gavage feeding, may be necessary for the first weeks to months of life to assure adequate nutrition and growth, and to prevent failure to thrive. Gastrostomy tube placement is rarely necessary as feeding improves with time, but could be considered if clinically indicated. The

hyperphagia is believed to be caused by a hypothalamic abnormality resulting in lack of satiety. However, the exact pathophysiology and molecular mechanism underlying excessive eating or hyperphagia remains unclear. Several studies have suggested the elevated levels of ghrelin, a potent circulating orexigenic hormone, correlate with the onset of hyperphagia in individuals with PWS.^{66,67} But other studies have not reached the same conclusion.^{68,69} Interestingly, the prohormone convertase PC1 (encoded by PCSK1) is reduced in PWS patient iPSC and iPSC-derived neurons as well as in the PWS mouse model with deficiency of *Snord116*.^{70,71} Because deficiency of PC1 in humans and mice causes the hyperphagic obesity, the result of reduced PC1 may support that the major neuroendocrine features in PWS are the result of PC1 deficiency. No known medications help control excessive eating or suppress food seeking behavior for individuals with PWS. Behavioral modification and food access restriction remain the primary intervention.⁷² Short stature is common in PWS, and growth hormone treatment is the mainstream therapy for PWS clinical management.^{12,73,74} There have been extensive investigations into whether PWS patients have growth hormone deficiency, although study quality varies widely. In the literature, the reported prevalence of growth hormone deficiency in patients with PWS ranges from 40% to 70%.¹³ Factors contributing to this significant discrepancy remain unclear, and a varying percentage of subjects show stimulated growth hormone values within the normal range. More importantly, the mechanism underlying growth deficiency associated with the deficiency of paternally expressed genes is completely unknown. Nevertheless, growth hormone has been widely used in clinic to treat PWS.⁷⁵ The bulk of evidence demonstrates benefit from growth hormone treatment therapy, including improvement in final height, body composition, physical strength, increased lean body mass, decreased fat mass, and increased mobility. Several small-scale studies also report the positive findings in cognitive and behavioral impairments in PWS patients.^{76,77} However, systemic investigation is necessarily needed to determine whether growth hormone therapy also improves cognitive function and helps in the management of behavioral problems. Recently, multiple clinical trials indicate that intranasal oxytocin treatment may have significant clinical benefits.⁷⁸⁻⁸¹ The mechanism underlying the clinical efficacy of oxytocin treatment remains to be elucidated.

Mouse models for PWS.

The 15q11-q13 region in humans is highly conserved in the central chromosome 7C region in mice. The structures of the imprinting domain, imprinted expression for most genes, and IC are also conserved in mice.⁸² The exception to these is that the long noncoding RNAs of *SPA1* and *SPA2* in human are apparently not conserved in rodent.⁵² Researchers have produced and characterized a series of genetically modified mouse models with inactivating mutations at different genes or loci within the mouse central chromosome 7 (Table II). Until recently, it has been difficult to evaluate the validity of these models, as precise genotype and phenotype correlations between genetic defects in different genes in the 15q11-q13 regions and the features of PWS in humans remained unclear. It has become evident in recent years that *SNORD116* deficiency is critical to the key PWS clinical features. However, other paternally expressed genes may also modify the clinical presentation with a large paternal deletion, maternal UPD, and imprinting defect. For comparison with the majority of human PWS cases, an ideal model for PWS would be genetically mutant mice carrying a chromosome deletion that is homologous with either a type I or type II deletion

in human 15q11-q13 region. To date, there are no reports of such a model. We have attempted to engineer a mouse chromosome with a deletion from *Gabrb3* to *Ndn* and *Ube3a* to *Ndn*, but have failed to obtain germline transmission despite successful introducing these deletions into mouse embryonic stem cells (Jiang and Beaudet, unpublished data). A virus-induced transgene insertion in the 7C of the mouse chromosome 7 (Tg^{PWS/del}) has been reported and characterized.⁸³ The exact breakpoint and size of the deletion has not been delineated, but is estimated to span the region between *Ndn* and *Ube3a*. Unfortunately, this mouse line is no longer available for further characterization.⁸² Researchers have used a Cre/*loxP* chromosomal engineering technique to generate and characterize mice carrying a ~500 kb deletion from exon 1 of *Snprn* to exon 2 of *Ube3a*, which includes the cluster of *Snord116* in the paternal chromosome.⁸⁴ The paternal deletion results in a highly penetrant perinatal lethality and poor feeding, which is considered to recapitulate human neonatal and early infant PWS phenotypes. Smaller deletions restricted to *Snord116* are also reported.^{85,86} Mouse phenotypes of the *Snord116* paternal deletion are similar to, but milder than, those of mice with a paternal deletion from *Snprn* to *Ube3a*. Mutations in the individual, paternally expressed genes *Snprn*, *Ndn*, and *Magel2* are also reported. No significant phenotypes have been associated with paternal deficiency of *Snprn*.⁸⁴ In *Ndn* mutant mice, perinatal lethality has been associated with 1 line⁸⁷ but not another.⁸⁸ Extensive phenotypic analyses have been conducted for 2 different lines of paternal *Magel2* deficient mice.^{89,90} Both prenatal and postnatal partial lethality have been reported in these lines. Multiple lines of mutant mice with small deletions in the region homologous to that in human PWS—IC have also been generated and characterized.⁹¹⁻⁹³ Similar to other lines of mutant mice with deletion in paternally expressed genes, partial perinatal lethality is a common feature associated with these IC mutant lines. Discussion of the detail phenotypes for each mutant mouse line is beyond the scope of this review, and readers are referred to updates in the literature and recommendations of the PWS Animal Models Working Group.⁸² One of the most intriguing findings from a review of all PWS-related models is that none of mutations in germline recapitulate obesity phenotype despite some models may display some degree of hyperphagia phenotype. Interestingly, Cre-expressing adeno-associated virus was used to generate mice with a conditional deletion of *Snord116s* in the mediobasal hypothalamus; these animals became hyperphagic between 9 and 10 weeks after injection, and a subset developed marked obesity.⁹⁴ The underlying cause of this discrepancy between germline and conditional deletions of *Snord116* remains unclear and warrants further investigation.

Epigenetic therapy for PWS.

Epigenetic therapy refers to the use of drugs or other epigenome-influencing techniques for the treatment of human diseases. Over the last 2 decades, this approach has been investigated for the development of novel cancer treatments.⁹⁵⁻⁹⁸ It is beyond the scope of this review to summarize this progress of cancer epigenetic therapy. Search of [Clinicaltrials.Gov](https://clinicaltrials.gov/) (<https://clinicaltrials.gov/>) using the combined terms “Cancer” and “Epigenetics” returned a total 207 clinical trials (completed and active), 73 of which are actively recruiting. Epigenetic dysregulations at different levels are wide spread in cancer cells.^{97,99} These could be primary, such as global or gene-specific hyper- or hypomethylation of DNA, alterations in the histone modifications, or secondary to mutations in genes that encode epigenetic proteins.⁹⁹ The development of epigenetic cancer therapy are mostly focusing on

identifications of small molecules and compounds with the capacity to reverse the epigenetic changes found in cancer cells.^{97,100} These include targeted therapies for a specific type of epigenetic dysregulation and broad epigenome reprogrammers. While almost all types of epigenetic modifications are drug development target candidates, DNA methylation, and histone acetylation have gained the most attention.¹⁰¹ Broad epigenome reprogrammers such as inhibitors for DNMTs and HDACs have been tested in cancers and several have obtained the Food and Drug Administration (FDA) approval for use in humans.¹⁰²⁻¹⁰⁵ These drugs tend to cause large-scale changes in gene expression, generally reversing cancer-specific expression. A long list of other compounds targeting various histone and chromatin modifications is at different investigational stages.¹⁰⁰ Targeted epigenetic therapies are focusing on specific epigenetic changes in a subset of cancer patients based on the knowledge of the disease expression signature. For example, The H3K27 histone *N*-methyltransferase EZH2 is activated by mutations in lymphomas,¹⁰⁶ and use of an EZH2 inhibitor induced selective killing of cell lines carrying such mutations.¹⁰⁷ The knowledge gained from these epigenetic cancer therapies will certainly be applicable and contribute to the development of similar drugs for brain disorders.¹⁰⁸

As genomic imprinting disorders have unique molecular and epigenetic defects, they offer hopeful opportunities to explore epigenetic therapy in humans. For example, the genes responsible for AS and PWS are expressed from either the maternal or paternal chromosome but are repressed or silenced in the corresponding paternal or maternal chromosome. The genetic structure of AS and PWS genes in the silenced chromosome 15q11-q13 remain intact, but are transcriptionally repressed via an epigenetic mechanism. Thus, the hypothesis for epigenetic therapy in these disorders is straightforward; namely, a pharmacologic approach would utilize epigenetic modification to reactivate expression of the repressed AS and PWS genes. This hypothesis is supported by early studies that treatments of cultured PWS patient-derived cells with DNA methylation inhibitors of 5-Azacytidine and 5-aza-2'-deoxycytidine result in the reactivation of the paternally-expressed *SNPRN* gene on the maternal chromosome.^{41,109} Similarly, but less consistently, treatment with an HDAC inhibitor has also been shown to reactivate expression of the *SNRPN* gene in PWS patient cells.^{41,109} The effects of treatment with DNA methylation inhibitors in vivo has not yet been reported. Nevertheless, these observations support the theory that either DNA methylation or histone modification contributes to PWS gene repression in the maternal chromosome.

To systematically identify new drugs or small molecules capable of reactivating AS and PWS gene expression, Huang et al conducted a high context small molecule screening in cultured neurons derived from mice carrying a UBE3A-YFP fusion protein transgene^{110,111} (Fig 3, A and B). Screening of 2306 small molecule compounds identified a list of both topoisomerase I and II inhibitors capable of reactivating expression of the *Ube3a* gene on the paternal chromosome, both in cultured neurons and in vivo in mouse brains. One of topoisomerase I inhibitors, topotecan, appears to reactivate *Ube3a* expression and is FDA approved for use as a chemotherapy drug.¹¹² For this reason, topotecan has been more extensively studied in the AS mouse model.¹¹⁰ Considering the known toxicity of current topoisomerase inhibitors, the prospect of their use in human AS has not been fully investigated but is expected to be low. Recently, new derivative of topoisomerase I inhibitor

that are capable of reactivating the expression of *Ube3a* from the paternal chromosome in cultured neurons have been reported.¹¹³ These modified new compound may offer a new opportunity to test them in vivo as well as to assess the toxicity of these compounds.

A similar strategy has been applied to investigate *SNORD116*, the key PWS candidate gene.¹¹⁴ *SNORD116* is a noncoding small nucleolar RNA that is processed from the intron of a continuous noncoding RNAs presumably initiated from the promoter of *SNRPN*.^{53,115} As *Snord116s* are noncoding RNAs, it is not feasible to design a direct high content screening approach. Investigators have performed high-content small molecule screening using mouse embryonic fibroblast (MEFs) derived from the maternally inherited *Snrpn-EGFP* fusion transgene^{114,116} (Fig 3, C and D). Expression of *Snrpn-EGFP* in MEFs and the brain was confirmed by mRNA analyses. The working hypothesis is that small molecules that reactivate expression of *Snrpn* from the maternal chromosome should have the same effect on *Snord116s*. Screens of more than 9000 small molecules from 13 libraries have identified a list of G9a/EHMT2 inhibitors, primarily UNC0642 and UNC0638, that are capable of reactivating the expression of SNRPN-EGFP in MEFs.¹¹⁴ As hypothesized, both UNC0642 and UNC0638 can reactivate *SNORD116* expression in mice in vivo as well as in cultured fibroblasts derived from PWS patients with a paternal deletion of 15q11-q13. Most importantly, treatment with UNC0642 during postnatal days 5–12 has demonstrated the same reactivation of *Snrpn* and *Snord116*, which in turn was protective against perinatal lethality and poor feeding in the PWS mouse model carrying a paternal deletion from *Snrpn* to *Ube3a*.^{84,114} The same treatment in wild type mice did not cause any significant acute toxicity at the given dose. The treatment is also effective in adult mice to reactivate the expression of SNRPN-EGFP fusion transgene in brain and liver from the maternal chromosome.¹¹⁴ It remains to be investigated whether the treatment could have the same effect for the human neurons derived from the iPSCs of PWS patients. These results provide the first proof-of-principle for the feasibility of epigenetic therapy in PWS (Fig 4). As expected, treatment with a G9a/EHMT2 inhibitor reduced the H3K9me2/H3K9me3 in PWS—IC and multiple other loci within the imprinted domain. The chromatin accessibility assay also indicates that the treatment of G9a results in a more open chromatin in PWS imprinting domain that supports a chromatin spreading model in response to the change of H3K9me2.¹¹⁴ An interesting question to be investigated is whether the treatment of EHMT2/G9a inhibitor may also alter the expression of paternally expressed PWS genes on the paternal chromosome. One unexpected, but interesting, finding at a mechanistic level is that treatment with G9a/EHMT2 inhibitors did not change DNA methylation in the PWS—IC, yet still reactivated paternally-expressed genes in the maternal chromosome. This suggests that the PWS—IC mechanism that is responsible for imprinted expression of PWS genes operates independently of DNA methylation. It also raises the question as to whether combined DNA methylation inhibitors and histone modulators would have a synergistic effect and raise the efficiency of PWS gene reactivation. In assessing the prospective of clinical application of EHMT2/G9a mediated epigenetic based therapy, it is expected that the EHMT2/G9a inhibitor treatment would be effective for PWS patients caused by all 3 major classes of defects (large paternal deletion, maternal UPD 15, and imprinting defect). However, further validation in different model may be warranted.

The impact with a G9a/EHMT2 inhibitor on other paternally expressed genes in the 15q11-q13 region.

As diagramed in Fig 2, *NDN*, *MAGEL2*, *MKRN3*, and *SNORD115s* are other paternally expressed genes or transcripts that are mapped in the imprinted domain. The interesting and also important question for future translational research is whether EHMT2/G9a inhibitors are also capable of reactivating these genes from the maternal chromosome. Treatment with the G9a/EHMT2 inhibitor can reactivate the expression of host transcript of *SNORD115s* and *NDN* in cultured fibroblasts from PWS patients with a paternal deletion of 15q11-q13.¹¹⁴ Reactivation of *MAGEL2* has not been determined in cultured fibroblasts because of its brain-specific expression pattern.¹¹⁷ Further investigation into reactivation of *Ndn*, *Mkrm3*, and *Magel2*, and *Snord115s* by G9a/EHMT2 inhibitors should be performed *in vivo* including brains of mutant mice carrying the paternal deficiency of these genes. The positive results will not only shed the insight for the mechanism underlying the EHMT2/G9a treatment but also provide the evidence to support translational potential for the individuals carrying mutations only in *MAGEL2* and *MKRN3* in clinic.

The impact of epigenetic therapy for PWS on the AS *Ube3a* gene.

There is ample evidence from genetically modified mice studies that antisense RNA of *Ube3* (*Ube3a-ATS*), which also initiates from the IC region of the paternal chromosome, is responsible for brain-specific imprinted expression of AS *Ube3a*.^{118,119} This raises the question of whether use of a G9a/EHMT2 inhibitor to reactivate PWS genes in the maternal chromosome would also activate *Ube3a-ATS*. Treatment with an EHMT2/G9a inhibitor does not affect *Ube3a-ATS* and *UBE3A* expression in the mouse brain,¹¹⁴ but does reactivate the expression of host transcripts of *SNORD116* and *SNORD115*. The transcriptional mechanisms underlying the generation of long noncoding host transcripts for *SnoRNAs* and *Ube3a-ATS* from the interval between PWS—IC and *Ube3a* are not well understood. Studies using EHMT2/G9a inhibitors suggest that expression of *Snord116* host transcript and *Ube3a-ATS* are regulated differently. It is possible that these differences arise at the transcriptional start sites for the host transcripts of *Snord116* and *Ube3a-ATS*.⁵⁴ G9a/EHMT2 inhibitors affect the transcription start site for the host transcripts of *Snord116*, but not the *Ube3a-ATS*. Genome-wide H3K9me profiling indicates that the continuous distribution of H3K9me2 along the PWS domain does not extend to the gene body of *UBE3A*.¹²⁰ In this case, the effect of the G9a/EHMT2 inhibitor weakens as it becomes more distal to the PWS—IC. This is because a domain-specific chromosome insulator close to *UBE3A* defines H3K9me2 in the PWS region.¹²⁰

Other potential targets for epigenetic based therapy.

The PWS—IC and PWS imprinting domain is known to be differentially modified by multiple epigenetic modifications. shRNA-mediated knock down of *SETDB1*, a methyltransferase responsible for the H3K9me3, in PWS patient-derived iPSCs and iPSC-derived neurons results in partial reactivation of *SNORD116* and the host transcripts.¹²¹ The change of H3K9me3 is associated to only with *SNORD116s* locus but not in PWS—IC. The primary change associated the treatment of EHMT2/G9a inhibitors is the reduced H3K9me2 in both *SNORD116s* and the PWS—IC.¹¹⁴ Reduction of H3K9me3 associated with

*SNORD116*s is also observed in cells treated with EHMT2/G9a inhibitor. In contrast to the G9a/EHMT2 inhibitors, inactivation of SETDB1 reduces DNA methylation in the PWS—IC despite no change of H3K9me3. The SETDB1 interacts with ZNF274, a zinc finger protein.¹²² In PWS patient iPSCs-derived neurons, inactivation of *ZNF274* using a CRISPR/Cas9 method also reduces H3K9me3 in the PWS—IC and reactivates *SNRPN* and *SNORD116* clusters from the paternal chromosome.¹²³ Interestingly, not all *SNRPN* transcripts were reactivated as a result of *ZNF274* inactivation. Similar to G9a inhibitors, but different from the *SETDB1* knockdown, *ZNF274* deficiency does not affect the DNA methylation of the PWS—IC. These results together suggest that distinct mechanisms may underlie reactivation of paternally expressed genes from the maternal chromosome mediated by EHMT2/G9a and SETDB1/ZNF274. Nevertheless, these findings together support that SETDB1 and ZNF274 are druggable targets for the development of molecular therapy of PWS.

Genome-wide off-target effects or/and epigenetic toxicity related to epigenetic therapy.

Common concerns regarding the use of epigenetic drugs include the potential for genome-wide or off-target effects that may be referred as epigenetic toxicity. While this is logical in concept, the EHMT2/G9a inhibitor has been well tolerated in animal studies, suggesting that evaluations of epigenetic toxicity should be conducted on a case-by-case basis with careful consideration of the dose, duration, route, and timing of the drug delivery. One example of epigenetic drug safety in practice is the tolerability of an FDA approved DNA methylation inhibitor used in the treatment of myelodysplastic syndrome patients.^{102,124} It is theorized that an epigenetic inhibitor, such as the EHMT2/G9a inhibitor, could generate genome-wide up- or down-regulation of hundreds of genes. Genome-wide mRNA expression profiling of animals treated with different dosing, duration, and at different ages may provide better assessment of toxicity. However, it is difficult to determine whether these changes would reach the threshold of physiological toxicity. It would be even more difficult to predict whether the net impact of up- or down gene regulation is a direct result of the epigenetic drug treatment. In the case of G9a/EHMT inhibitors, genome-wide ChIP analysis for targeted H3K9me2 may help to assess the molecular impact and correlation with change of gene expression. However, the challenge may remain to assess the net impact of physiological interaction among the genes that are dysregulated by epigenetic level in vivo and in long term. To elaborate on this, there is one noteworthy example in humans in which disruption of multiple imprinted loci due to germline mutations in *ZFP57* resulted in only a relatively mild condition, a rare case of transient neonatal diabetes.^{125,126} These observations suggest that evaluating the broad effects of epigenetic drugs is the more complex task at hand and the molecular targeting specifically to SETDB1 or ZNF274 may be an alternative approach to explore in future study.

Concluding remarks and future directions.

PWS is a life-long debilitating genetic and epigenetic condition that has a significant impact on quality of life. The molecular basis underlying PWS is unique and presents a hopeful opportunity to explore epigenetic-based molecular therapy. Broad evidence from PWS patient-derived cellular models and PWS mouse models support the proof of principle that epigenetic modifications to reactivate PWS genes from the maternal chromosome 15q11-q13. Inhibition of H3K9me2 with small molecules derived from high content screening is

capable of reactivating *SNORD116* expression, a critical gene for PWS, and protects against perinatal lethality and failure of thrive in 1 PWS mouse model. The discovery that inactivation of SETDB1 and ZNF274 that primarily involve H3K9m3 can also reactivate the imprinted expression of PWS genes support the additional epigenetic targets for the future development of PWS therapies. Future investigations need to assess the translational potential of therapy for these approaches in humans.

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Fig 1.

Molecular bases of PWS. Four types of molecular defects found in PWS are diagrammed. Chromosome ideogram in pink color represents the maternal origin and in blue color for paternal chromosome. The white strips represent the large or small deletion in the chromosome 15q11-q13. (M, maternal; P, paternal; IC, imprinting center; UPD, uniparental disomy). Type I: Large 6 Mb deletion in the paternal chromosome 15q11-q13. Type II: Maternal UPD of 15. Type III: Imprinting defect that with or without a microdeletion in the PWS—IC. The IC defect causes the maternal epigenotype in the 15q11-q13 region in the paternal chromosome measured by DNA methylation. Type IV: Microdeletion in the region of *SNORD116s*. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig 2.

Human chromosome 15q11-q13 imprinting domain and homologous region in the mouse central chromosome 7C. The genes in blue color are expressed exclusively from the paternal chromosome. The gene in pink color is expressed exclusively from the maternal chromosome. The genes in black color are expressed from both maternal and paternal chromosomes. The imprinted expression pattern for *PWRN1*, *NPAP1*, and *UBE3A* is tissue or cell type specific (BP, breakpoint; IC, imprinting center). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig 3.

Drug screening strategy to discover the small molecule drugs for the epigenetic therapy in the PWS and AS. A, UBE3A-YFP fusion protein transgenic mice. B, Drug screening strategy using cultured neurons derived from UBE3A-YFP fusion protein mice to screen small molecule drug that can reactivate the expression of *Ube3a* from the paternal chromosome. C, SNRPN-EGFP fusion protein transgenic mice. D, Drug screening strategy using mouse embryonic fibroblasts (MEFs) derived from the maternal SNRPN-EGFP mice to screen small molecule drug that can reactivate the expression of *Snrpn* from the maternal chromosome.

Fig 4.

A proof-of-principle of epigenetic therapy for PWS by G9a inhibitors. G9a inhibitor of UNC0638 and UNC0642 (block dots) directly reduce H3K9 methylation (red stars), but do not change methylation of PWS—IC (black hexagon). The reduction of H3K9 methylation is shown to be sufficient to activate the expression of PWS genes from the maternal chromosome, thereby offering therapeutic benefits. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table I.

Clinical problems at different ages

<i>Birth to age 2 years</i>	
•	Hypotonia with poor suck (neonatal period) <i>Age 2—6 years</i>
•	Hypotonia with history of poor suck
•	Global developmental delay <i>Age 6—12 years</i>
•	Hypotonia often persists
•	Global developmental delay
•	Excessive eating with central obesity if uncontrolled <i>Age 13 years to adulthood</i>
•	Cognitive impairment, usually mild intellectual disability
•	Excessive eating with central obesity if uncontrolled
•	Hypothalamic hypogonadism and/or typical behavior problems

Modified from Driscoll et al.¹²

Table II.

PWS mouse models that can be used to test the novel therapy

Mouse strain	Description	Major phenotypes	References
pat-Del (Snrpn-Ube3a)	~500 kb Deletion from Snrpn to Ube3a	Perinatal lethality/poor feeding Postnatal growth retardation No obesity	84
pat-Del (Snord116)-Brosius	~deletion of IPW and Snord116	Perinatal lethality Postnatal growth retardation No obesity	85
pat-Del (Snord116)-Francke	~deletion of Snord116	Perinatal lethality Postnatal growth retardation Abnormal feeding Lean body composition No obesity	86
PWS-IC del 35 kb	35 kb deletion in the region homologous PWS-IC	Perinatal lethality Postnatal growth retardation No obesity	92
PWS-IC del 6 kb	6 kb deletion in the region homologous PWS-IC	Perinatal lethality Postnatal growth retardation No obesity	93
PWS-IC del 4.8 kb	4.8 kb deletion in the region homologous PWS-IC	Perinatal lethality Postnatal growth retardation No obesity	91
Magel2-Waverick	deletion of Magel2	Prenatal lethality Viable postnatally Postnatal growth retardation Abnormal food consumption Increased fat to muscle No obesity	89
Magel2-Muscatelli	deletion of Magel2	Postnatal lethality/poor feeding Postnatal growth retardation Abnormal food consumption Increased fat to muscle No obesity	90

Modified from Table I from Resnick et al.⁸²