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Perspective Series

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Introduction

Genomic imprinting, a process that causes genes to be expressed according to their parental origin, affects a minority of human genes, probably less than 1,000. Nevertheless, this class of epigenetic modification acts on many genes with critical roles in growth and development, and disordered imprinting is implicated in genetic disease and in many cancers. Various theories have been proposed to explain the evolution of imprinting in mammals. The most popular is the Haig parental conflict model, which holds that imprinting evolved as a result of opposing interests of the maternal and paternal genomes. Thus, in polygamous species, paternally derived genes will favor fetal growth at the expense of depleting maternal resources and disadvantaging further offspring. In contrast, the maternal genes will oppose the paternal effect and conserve resources to ensure the fitness of the mother and future offspring. This model predicts that although paternally expressed imprinted genes should promote growth, maternally expressed genes should have opposite effects. Many, but not all, imprinted genes identified to date conform to these predictions (1).

The mechanisms by which imprinting is established and maintained are the subject of intense investigation (2). The process must involve a reversible epigenetic marking, and a number of features of imprinted genes have been described. Thus, differential methylation of maternal and paternal alleles is a major characteristic of imprinted genes, and defects in methylation processes (e.g., methyltransferase deficiency) disrupt normal genomic imprinting. Parental allele-specific alterations in the chromosome environment of imprinted genes are revealed by the presence of asynchronous DNA replication and differences in chromatin structure and modification (e.g., histone acetylation).

A striking feature of imprinted genes in mammals is their tendency to cluster in the genome. The arrangement of coordinately or oppositely imprinted genes within a cluster offers insights into the mechanisms by which cells establish and maintain appropriate imprints on functionally related genes. Here, we discuss the organization and function of a major imprinted gene cluster, occurring on human chromosome 11p15.5, that has been implicated in the imprinting disorder Beckwith-Wiedemann syndrome (BWS) and in a variety of human cancers including Wilms' tumor (3, 4).

Because the regulation of imprinted genes in the homologous region in the mouse (distal region of chromosome 7) is broadly conserved between mice and humans, experiments in mice have complemented molecular analyses of BWS pathology and have contributed greatly to our current knowledge of mammalian genomic imprinting mechanisms. In this review, we consider the mechanisms and role of genomic imprinting in human development and in BWS pathogenesis, drawing on research on the human and the mouse clusters of imprinted genes.

Clinical genetics

BWS is a congenital overgrowth syndrome characterized by pre- and postnatal overgrowth, macroglossia, and anterior abdominal wall defects. Additional, but variable, complications include organomegaly, hypoglycemia, hemihypertrophy, genitourinary abnormalities, and, in about 5% of children, embryonal tumors (most frequently Wilms' tumor) (3). The genetics of BWS are complex, but evidence for genomic imprinting effects is present for each of the 3 major subgroups of patients (familial, sporadic, and those with chromosome anomalies) (see ref. 3 and references cited therein). Familial clustering accounts for approximately 15% of BWS cases. The great majority of sporadic and familial cases show no cytogenetic abnormality, but approximately 2% carry duplications, inversions, or translocations affecting distal 11p. Because BWS pathogenesis depends, in each of these subgroups of patients, on parent-of-origin effects, it appears that imprinting of 1 or more genes in this region is disrupted. Thus, in familial cases, the risk to offspring depends on the sex of the transmitting parent, with maternal transmission associated with greatly increased penetrance. Approximately 20% of sporadic cases demonstrate paternal uniparental disomy (UPD) for chromosome 11p15.5, and many other sporadic cases show altered imprinting of BWS candidate genes (see below). To date, all cases of UPD in BWS have resulted from postzygotic mitotic recombination and are mosaic for paternal isodisomy. Among the patients with BWS who have cytogenetic abnormalities — chromosome 11p15.5 duplications, inversions in distal 11p, or balanced translocations that break in this region — genomic imprinting effects are evident in the parental origin of the affected chro-

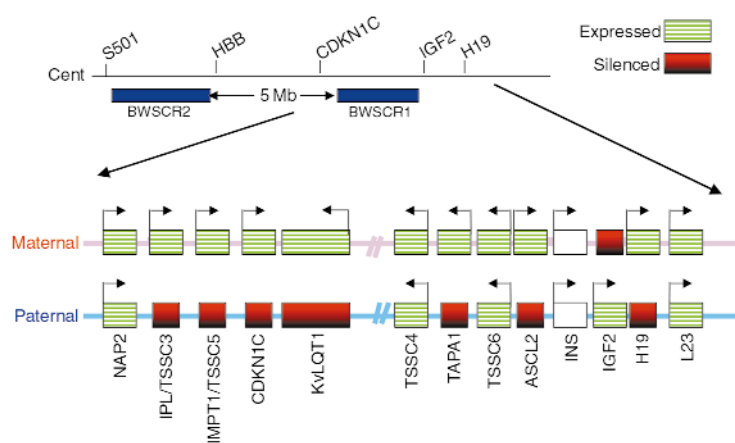


Figure 1

The imprinting cluster on human chromosome 11p15.5. The distal breakpoint cluster region (BWSCR1) is indicated. Breakpoints within BWSCR1 disrupt *KvLQT1*. Solid red symbols indicate allelic silencing; green/white symbols indicate allelic transcription. The imprinting status of the *INS* (insulin) gene has not been defined in humans. Further details are included in the text.

mosomes. Duplications in this group of patients are invariably derived from the patient's father, whereas inversions and translocations always involve the maternal homologue.

Maternally inherited rearrangements associated with BWS have been mapped to 3 distinct breakpoint cluster regions (BWSCRs). The most frequent of these is BWSCR1, and breakpoints in this region interrupt the *KvLQT1* (*KCNQ1*) gene and map at least 200 kb proximal to *IGF2* (Figure 1) (5). In addition, 2 less-frequent breakpoint cluster regions, BWSCR2 and BWSCR3, map approximately 5 Mb and 7 Mb centromeric to BWSCR1 (6).

Chromosome 11p15.5 and BWS candidate genes

A variety of strategies have been used to isolate genes from the imprinted gene cluster between *hNAP2* and *L23* on chromosome 11p15.5 (Figure 1). Initially, all genes identified from this region were imprinted, but at least 2 apparent exceptions, the nonimprinted *TSSC4* and *TSSC6* genes, are now known (7). These genes appear to subdivide the imprinted cluster region into 2 major subgroups, with proximal (containing *CDKN1C*, *KvLQT1*, *IPL/TSSC3*, and *IMPT1/TSSC5* genes) and distal (containing *IGF2*, *INS*, and *H19* genes) clusters separated by nonimprinted genes. This observation may oversimplify matters, as some genes are subject to partial, tissue-specific, or developmental stage-dependent imprinting.

Several chromosome 11p15.5 genes may be implicated in familial and sporadic forms of BWS. The most intensively studied of these candidates are *CDKN1C*, *H19*, *IGF2*, and *KVLQT1* (*KCNQ1*). *CDKN1C* encodes a cyclin-dependent kinase inhibitor belonging to the CIP family of cell-cycle regulators. Overexpression of *CDKN1C*, also known as p57^{KIP2}, can arrest cells in G1. Cells that have exited the cell cycle express this protein, and 2 germline *CDKN1C* mutations found in patients with BWS cause loss of cell-cycle inhibition (8). *H19* encodes an untranslated RNA of unknown function (but see the Perspective by Tycko in this series for a discussion of *H19* in tumorigenesis). *IGF2* encodes a fetal growth factor, and overgrowth in BWS is restricted to tissues in which *IGF2* is expressed. *KVLQT1* (*KCNQ1*) encodes a potassium channel that is mutated in long QT syndrome (a dominantly inherited cardiac conduc-

tion defect) and in Jervell-Lange-Nielson syndrome (an autosomal recessive disorder characterized by deafness and cardiac conduction defects) (9). Although *KVLQT1* is disrupted by BWSCR1 breakpoints, the *KVLQT1* gene product does not appear to have a role in BWS. However, *KVLQT1* encodes multiple transcripts, and a role for an untranslated transcript in imprinting cannot be excluded.

The *IGF2* and *H19* genes are closely linked and oppositely imprinted. The relationship between *IGF2* and *H19* imprinting has been investigated intensively in mouse experiments (see ref. 10 and references cited therein). The most common explanation for reciprocal imprinting of *IGF2* and *H19* postulates enhancer competition between these genes. According to this model, a shared enhancer downstream of *H19* drives *H19* RNA expression from the maternal allele. On the paternal allele, however, methylation of the *H19* promoter allows the enhancer to interact instead with the adjacent *IGF2* gene. Recently, an alternative model has been suggested in which an epigenetic mark upstream of *H19* acts as a boundary element on the unmethylated maternal chromosome (11). The imprinting of other genes within the cluster, such as *Cdkn1c* and *Kvlt1*, appears to be independent of *H19* imprinting (12).

Molecular pathology of BWS

The observation that paternal UPD causes BWS is consistent with the existence, on 11p15.5, of either paternally expressed genes that act as growth promoters or maternally expressed genes that suppress growth. As discussed later here, genes of both descriptions may be found within this chromosomal region, and there is strong evidence for both of these etiologic models. Because the entire BWS candidate region has been included in the disomic segment of all known UPD-associated cases, it has not been possible to reduce the range of candidate genes by analyzing these individuals. However, a subset of sporadic and familial BWS cases carry germline mutations in *CDKN1C*, providing unequivocal evidence for the involvement of a single gene in at least some affected people (13). Among familial cases, approximately 40% carry mutations in *CDKN1C*, but the incidence of such mutations among sporadic cases is much lower, at approximately 5% (14). *CDKN1C* is maternally

expressed, with a low level of expression from the paternal allele in humans but not in mice. Thus, the parent-of-origin effects on penetrance described in familial BWS are consistent with predominantly maternal allele expression of *CDKN1C*.

Although mutations in *CDKN1C* may be sufficient to cause disease in some individuals, the absence of such mutations in most sporadic cases of BWS indicates that other pathogenic mechanisms must exist. The paternally expressed fetal growth-promoting gene *IGF2* has long been considered a strong candidate for BWS. Two groups have reported that abnormal, biallelic expression of *IGF2* (often designated as loss of imprinting, or LOI) occurs in the majority of sporadic BWS cases (15, 16). However, in a further series, the frequency of *IGF2* LOI was reported to be less than 10%, differences that may, in part, reflect variable patient selection criteria (17).

Epigenetic analysis of patients with BWS with LOI of *IGF2* indicates that at least 2 imprinting centers (BWSIC1 and BWSIC2) exist on 11p15.5 (17–19; see also Table 1). Mouse experimental data linking *Igf2* and *H19* imprinting had already implicated defects at an “imprinting center” in the misexpression of these imprinted genes (10). Likewise, a small subgroup of sporadic BWS cases shows biallelic expression of *IGF2* and silencing of *H19*, accompanied by a paternal methylation pattern on both chromosomes, suggesting that an imprinting control region (BWSIC1) is inactive in such individuals (18). Candidate regulatory regions proposed from experiments with mice include a CpG-rich site immediately upstream of *H19* and an intergenic region between *Igf2* and *H19* (11). The analogous region immediately upstream of the human *H19* gene, which is thought to be the site of the primary (gametic) *H19* imprint, is in fact biallelically methylated in this subgroup of patients with BWS. So this pathway for “LOI” of *IGF2* may be more accurately described, in a mechanistic sense, as a “gain of imprinting” at *H19*.

In another pair of imprinting disorders, familial Prader-Willi syndrome (PWS) and Angelman syndrome (AS) deletions of upstream untranslated exons of the *SNRPN* gene have helped to define imprinting control regions (20), but, to date, no germline deletions or mutations have been identified in patients with BWS who have BWSIC1 defects. However, some sporadic

PWS and AS cases with imprinting center defects do not have deletions and probably represent epigenetic errors (21). All BWSIC1 cases described to date are sporadic, and they may also represent similar “epimutations” (our unpublished observations).

Brown et al. observed LOI for *IGF2* in the presence of a normal *H19* epigenotype in a patient with BWS who had inherited a chromosome 11p15.5 inversion breakpoint in BWSCR1 (22). These data provided the first evidence of an additional, *H19*-independent mechanism for *IGF2* imprinting in humans and suggested that another imprinting center (BWSIC2) must exist at or centromeric to the breakpoint. Most sporadic patients with BWS show *IGF2* LOI and a normal *H19* epigenotype, suggesting a possible BWSIC2 defect. Consistent with this possibility, Smilnich et al. (19) recently reported that patients with BWS who have *H19* hypermethylation — and, hence, BWSIC1 defects — exhibited normal methylation patterns in a differentially methylated region (DMR) near the *KVLQT* gene (KvDMR1; see below). In contrast, patients with normal *H19* methylation patterns frequently showed loss of methylation on the maternal allele at KvDMR1 (19) (Figure 2). In 2 of 2 informative patients, KvDMR1 demethylation was associated with *IGF2* LOI and normal *H19* epigenotype. Loss of maternal allele KvDMR1 methylation correlated with expression of an antisense transcript, *KCNQ1OT*, from the maternal copy of this gene.

These findings are consistent with 2 imprinting control centers in 11p15.5: (a) a distal BWSIC1 regulating *IGF2* and *H19* imprinting and (b) a more centromeric BWSIC2 regulating the imprinting of *IGF2* and the expression of this antisense transcript. Lee et al. (17) reported similar results, but found that KvDMR1 demethylation was not associated with *IGF2* LOI in all cases, although it was consistently associated with biallelic expression of the *KCNQ1OT* transcript (which they term LIT1). Thus, BWSIC2 regulation of *IGF2* may be polymorphic, or LOI of *IGF2* may be sensitive to the exact nature of the BWSIC2 defect. One important issue that has not yet been resolved is the effect of BWSIC2 defects on maternal *CDKN1C* expression (Table 1). In the mouse, *Cdkn1c* expression requires a maternal germline imprint (23), although maternal germline methylation has not yet been found in or near the gene. Recent transgenic experiments have failed to identify a functional imprinting signal within 35 kb surrounding the *CDKN1C* gene (24). It would therefore not be surprising if *CDKN1C* imprinting were influenced by the BWSIC2, and a preliminary genotype-phenotype correlation is consistent with this hypothesis, as discussed later here.

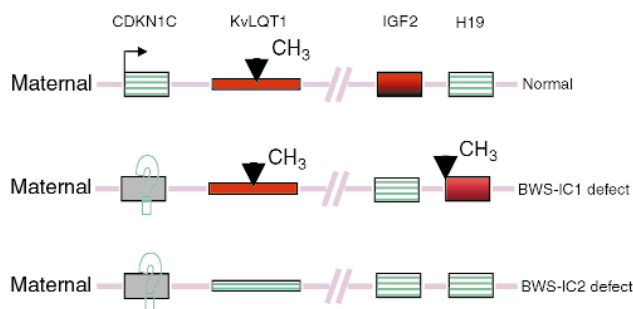


Figure 2

Maternal epigenotype in normal controls and in patients with BWS who have BWSIC1 and BWSIC2 defects. Solid red symbols indicate allelic silencing; green/white symbols indicate allelic transcription. CH₃ indicates methylation.

Mouse models of BWS and genotype-phenotype correlations

Several genetically engineered mouse strains have been developed that recapitulate aspects of the BWS phenotype and of BWS candidate gene regulation. Thus, overexpression of *IGF2* in transgenic mice causes overgrowth, macroglossia, and organomegaly in a dose-dependent fashion (25, 26). Murine *H19* deletions, likewise, mimic the epigenetic effects of

Table 1

Molecular subgroups of BWS: observed (in bold) and predicted (in italics) characteristics for *IGF2*, *H19*, and *CDKN1C*. (Data are derived from references 2, 13, 15, 17, 18, and 21.)

BWS subgroup	UPD	Maternal rearrangement	Paternal duplication	CDKN1C mutation	BWSIC1 defect	BWSIC2 defect
<i>IGF2</i> expression	<i>Increased</i>	Biallelic	<i>Increased</i>	<i>Unaltered</i>	Biallelic	Biallelic (variable)
<i>IGF2</i> methylation	Increased	Normal	Increased	Normal	Increased	Normal
<i>H19</i> expression	<i>Reduced</i>	Unaltered	<i>Unaltered</i>	<i>Unaltered</i>	Reduced	Unaltered
<i>H19</i> methylation	Increased	Normal	Increased	Normal	Increased	Normal
<i>KvDMR1</i> methylation	Reduced			Normal	Normal	Normal
<i>CDKN1C</i>	<i>Reduced expression</i>		<i>Unaltered</i>	Mutated	<i>Unaltered</i>	<i>Reduced?</i>
Replication timing	Altered	Altered			Normal	
Frequency	20% sporadic	2%	2%	40% familial 5% sporadic	5–10% sporadic	~40% sporadic

BWSIC1 defects (11, 18). Although the earliest definitive evidence for a second imprinting center (BWSIC2) arose from studies of patients with BWS, the observation that *H19* deletions do not affect imprinting of *Cdkn1c* and *Kvlqt1* in the mouse (12) provided independent data compatible with the existence of such a regulatory site. Third, a *Cdkn1c* knockout mouse, a model for BWS cases with germline *CDKN1C* mutations (27), displays anterior abdominal wall defects, renal medullary dysplasia, and adrenal cortical cytomegaly, but lacks other features of BWS, notably prenatal overgrowth. It remains to be established whether mouse and human fetuses respond fundamentally differently to lack of *CDKN1C*, or—perhaps more likely—whether the BWS mutations are not null alleles but the mouse knockout is. In any event, these findings suggest a link between exomphalos and *CDKN1C* inactivation in BWS. However, Eggenschwiler et al. (26), who developed yet another mouse model of BWS by crossing mice with *H19* and *Igf2r* mutations, identified another mechanism that may account for this phenotype. As a result of *Igf2* LOI (due to the *H19* deletion) and reduced clearance of circulating IGF2 protein, the resulting double knockout exhibited greater increases in levels of IGF2 than did the *Igf2* transgenic mice described here (25), and they displayed both overgrowth and exomphalos.

The variable molecular pathology of BWS provides scope for genotype-phenotype correlations. Analysis of uniparental disomy cases demonstrated a close association with hemihypertrophy (presumably resulting from differing degrees of mosaicism between the 2 sides). We have also defined a high incidence of exomphalos (9/11, 81%) in patients with BWS with germline *CDKN1C* mutations (14). Exomphalos is not a feature of patients with BWS who have UPD or BWSIC1 defects, and the absence of exomphalos in UPD cases probably reflects a milder phenotype because of mosaicism.

Phenotypic differences between *CDKN1C* mutation cases and patients with BWSIC1 defect strongly suggest that, although *IGF2* overexpression and *CDKN1C* inactivation may perturb the same signaling pathway, their effects are not equivalent, perhaps because *IGF2* and *CDKN1C* antagonize each other's effects, but only in certain tissues or at certain developmental stages. The genotype-phenotype correlations in BWS are broadly consistent with the differing phenotypes of the *IGF2* overexpression and *CDKN1C* knockout mouse models of BWS. In addition, we note that children with BWS with chromosome 11 duplications (predicted to have increased *IGF2* expression and normal *CDKN1C* expression) do not have exomphalos. Historically, anterior wall defects in BWS have been attributed to the mechanical effects of intra-abdominal organomegaly. However the close association between exomphalos and *CDKN1C* inactivation suggests that the developmental defect reflects alterations in cell growth, differentiation, or death in the abdominal wall. Preliminary genotype-phenotype analysis of BWSIC2 defect cases suggest that exomphalos is relatively common (E.R. Maher, unpublished observations). According to our model of the pathogenesis of exomphalos in BWS, this observation would predict altered *CDKN1C* expression in BWSIC2 cases.

BWS is genetically heterogeneous, and it appears that phenotypic differences between molecular subgroups reflect differential alterations in BWS candidate genes. To date, there is strong evidence for both *IGF2* and *CDKN1C* in the pathogenesis of BWS and these genes may be linked to particular features of BWS (e.g., exomphalos and *CDKN1C* inactivation; overgrowth and *IGF2* overexpression in mice and, possibly, in humans). The significance of *H19* in determining the BWS phenotype is unclear. A tentative association between reduced *H19* expression in BWS and Wilms' tumor susceptibility has been suggested (4). Our own data would be consistent

with this hypothesis, as BWS cases with Wilms' tumor have had UPD or BWSIC1 defects. However the numbers are too small to allow meaningful statistical comparison between subgroups, so this aspect requires further investigation.

The phenotype of the X-linked overgrowth disorder, Simpson-Golabi-Behmel syndrome (SGBS) overlaps with BWS. Many cases of SGBS result from defects in the glypican-3 (*GPC3*) gene (28). Although glypican-3 has been suggested to negatively regulate IGF2, direct evidence for this hypothesis is not available, and *GPC3* mutations may disrupt other signaling pathways (29). Nevertheless, the phenotypic similarities between BWS and SGBS suggest functional relationships between IGF2, *CDKN1C*, and *GPC3* gene products.

Molecular mechanisms of normal and abnormal imprint regulation

The mechanisms of introduction of imprints in germ cells, their maintenance in the embryo, and the reading of the imprints and their conversion into differential gene activity are still largely unknown (2, 10). However, a few elements have been defined that are important for the control of imprinting and that may account for the aberrant gene regulation in BWS. Apart from enhancers and promoters, 2 distinct, but possibly related, elements have been described that may contribute to allele-specific gene expression: antisense promoters and boundary elements. These elements are epigenetically regulated by DNA methylation or chromatin modifications.

Parental allele-specific methylation differences are a frequent, but not invariable, feature of imprinted genes; 11p15.5 includes several DMRs, including the paternally methylated sites in the *IGF2* gene (equivalent to mouse DMR2), the DMR upstream of *H19*, and the maternally methylated KvDMR1 site within the *KvLQT1* gene (17–19) (Figure 2). Untranslated antisense transcripts, a recently recognized characteristic of imprinted regions, are also found for multiple genes in the BWS region. An antisense transcript (*air*) from the murine *Igf2r* gene is expressed from the paternal allele, which bears a methylated promoter and is silent with respect to sense transcript expression. However, on the maternal allele, methylation of an intragenic site is associated with repression of the antisense transcript and expression of the sense *Igf2r* mRNA (30). Thus, although the relationship between sense and antisense expression remains obscure, antisense *Igf2r* RNA is linked with *Igf2r* imprinting in a process that has been termed expression competition (31). In addition, a *KvLQT1* antisense transcript (KCNQ1OT or LIT1) has been implicated in BWS (17, 19). How antisense transcription prevents sense transcripts is not clear.

A chromatin boundary element in the imprinting control region upstream of *H19* apparently regulates access of enhancers to nearby promoters (11, 32). Here, the demethylated boundary region restricts access of the *H19* endoderm enhancers, which are located 3' of *H19*, to its promoter. When methylated, the boundary is "open" and allows the enhancers access to *Igf2*. Although promoter methylation affects local gene expression, boundaries that open and close appear to contribute to broader regional controls.

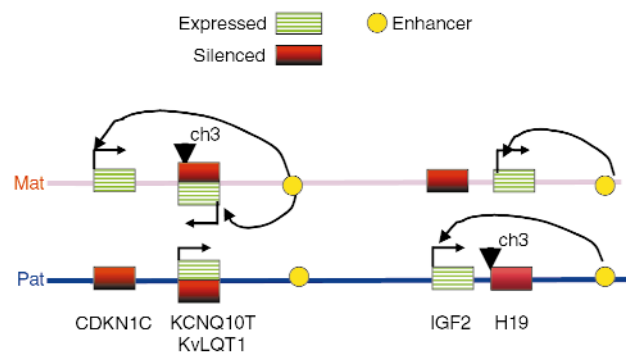


Figure 3

A speculative model for imprinting of genes in the BWS cluster on chromosome 11p15.5. Shared enhancers exist for *IGF2* and *H19* and are proposed for *CDKN1C* and *KvLQT1*. Methylation of the *H19* DMR silences *H19* and activates *IGF2*. Methylation of *KvDMR1* is proposed to silence *KCNQ1OT* and to activate *CDKN1C* and *KvLQT1*.

A very speculative model for gene regulation within the BWS cluster can be envisaged, using these building blocks (Figure 3). The KvDMR1 regulates antisense expression and is a boundary, which is open when methylated (as in the normal maternal chromosome). The proposed enhancers for both *CDKN1C* and *KvLQT1* are located 3' of this boundary. On the maternal allele, these enhancers are active and both *CDKN1C* and *KvLQT1* are expressed, consistent with the requirement for a maternal germline imprint for *CDKN1C* activity. The antisense transcript (KCNQ1OT) is made on the paternal allele and blocks paternal expression of *KvLQT1*. At the other end of the cluster, methylation of the *H19* DMR on the paternal allele permits transcription of *Igf2* by an enhancer residing 3' of both genes. Methylation of *H19* on the maternal chromosome (e.g., in BWSIC1 defect cases) then leads to LOI of *IGF2*. In BWSIC2 defects, demethylation of KvDMR1 on the maternal chromosome (or BWSCR1 translocations) would be predicted, under this model, to lead to silencing of *CDKN1C*. How this could also lead to LOI of *IGF2* is not clear.

Imprinting defects in 2 principal locations (e.g., BWSIC1, BWSIC2) can explain the molecular pathology in a proportion of patients. However, it is likely that defects in other elements will be found in other classes of patients. In addition, levels of epigenetic information other than methylation can also be affected. It is now clear that one of the ways methylation can exert its repressive effects is through deacetylation of histones, which results in chromatin compaction that is less efficiently transcribed (33). Hence, defects in local or regional patterns of histone acetylation could also lead to altered expression of imprinted genes. This mechanism needs to be investigated in BWS.

Conclusions

Although the *IGF2* and *H19* genes are well characterized, the downstream effectors of IGF2 have not been elucidated in detail, and the function of *H19* RNA is enigmatic. It is reasonable to speculate that the link

between the functions of the IGF2 and CDKN1C gene products that are revealed by their involvement in BWS might turn out to be the first of many examples of functional interactions between genes within an imprinted gene cluster. Thus, clustering of imprinted genes enables coordinate regulation of imprinting across large domains and more local mechanisms within specific regions of the domain. Thus, on 11p15.5, BWSIC1 may provide a local mechanism for regulating IGF2 and H19 imprinting, whereas a second mechanism, possibly related to chromatin structure, regulates imprinting over a larger distance.

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