Imprinting errors and developmental asymmetry

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There are, in the broadest sense, two mechanisms by which gene expression can be extinguished in vertebrates. The first of these is based on mass action effects of positive and negative regulatory factors and is termed activation and repression; the second is independent of positive regulatory factors but is based on the history of the affected gene and is termed silencing. It can be said, again in the broadest sense, that imprinted genes, genes subject to X inactivation, and transposon promoters are subject to silencing, while the promoters of tissue-specific genes in non-expressing tissues are controlled by activation and repression. The escape of imprinted genes from silencing through unknown mechanisms can cause developmental abnormalities and can predispose to the formation of embryonal tumours. One developmental disorder caused by loss of imprinting of genes on chromosome 11p15.5 is Beckwith–Wiedemann syndrome (BWS). This syndrome has long been known to be inexplicably common in monozygotic twins; the twins are nearly always discordant for BWS, and nearly all twins are female. A loss of imprinting model based on stochastic errors in the nucleocytoplasmic trafficking of the DNA methyltransferase DNMT1, or a paternally expressed function that opposes maintenance methylation of maternally repressed growth-enhancing genes, is proposed to explain the perplexing genetics of BWS in monozygotic twins.

Keywords: imprinting; cytosine methylation; monozygotic twins

1. BECKWITH–WIEDEMANN SYNDROME AND GENOME IMPRINTING

BWS is a congenital condition that involves a predisposition to certain embryonal tumours (especially Wilms' tumour of the kidney) with overgrowth of abdominal viscera, macroglossia, omphalocele, and characteristic pits and creases of the earlobes (reviewed by Elliott *et al.* 1994). BWS is also associated with hemihypertrophy, in which structures on one side of the midline grow at a notably increased rate relative to tissues on the contralateral side. It has been known for many years that BWS is associated with monozygotic twinning (Berry *et al.* 1980); BWS is as common in monozygotic twins as in dizygotic twins, although dizygotic twins are about 10 times more common within the general population (Weksberg *et al.* 2002). Even more perplexing is the finding that BWS is nearly always discordant in pairs of identical twins, and that the affected twins are nearly always female, even though there is no other indication of sex-linked influences on BWS (reviewed by Junien 1992). These findings are clearly incompatible with any explanation based on Mendelian inheritance.

BWS is associated with the abnormal expression of imprinted genes within a complex gene cluster on human chromosome 11p15.5 (figure 1). A small subset of mammalian genes is subject to genomic imprinting, in which the state of expression of the two alleles in a diploid somatic cell is predicted only by the sex of the parent that

contributed the allele. The chromosome 11p15.5 region is currently believed to contain two adjacent but autonomously imprinted sub-domains: one that contains the *H19*, *IGF2* and *INS* genes, and a second that contains the *ASCL2* (formerly *MASH2*), *PHEMX* (*TSSC6*), *TSSC4* (*IMPT1*), *KCNQ1* (*KVLQT1*), *KCNQ1OT1* (*LIT1*, *KCNQ1AS1* or *KvDMR*), *CDKN1C* (*P57^{kip2}*), *TSSC3* (*IPL*), *CD81* (*TAPA1*) and *NAP114* (*NAP2*) genes. Each sub-domain is believed to contain an ICR. Imprinted expression of the *H19-IGF2* sub-domain depends on an ICR within a region 2–4 kb upstream of the *H19* gene. Deletion of this region eliminates normal imprinted expression (Thorvaldsen *et al.* 1998). Imprinted expression of the second sub-domain depends on an ICR that corresponds to the promoter region of the *KCNQ1OT1* gene, which encodes an unspliced and untranslated RNA that is transcribed from the antisense strand of part of the *KCNQ1* gene (figure 1). Deletion of this region also affected imprinted expression of all genes within the sub-domain but did not notably affect the *H19-IGF2* domain (Fitzpatrick *et al.* 2002). The fundamental mechanism of imprinted gene expression remains poorly understood, but is unlikely to be analogous to the mechanisms by which tissue-specific genes are activated and repressed during cellular differentiation.

Imprinted gene expression depends on patterns of methylated cytosine residues within imprinted domains (or sub-domains) that are established in the germline of the preceding generation. There is profound sexual dimorphism, not only in the identity of the genes affected (imprinting creates sexual asymmetries in gene expression) but also in the timing of imprint establishment. Maternal imprints are established in growing

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Figure 1. Imprinted sub-domains on human chromosome 11p and distal mouse chromosome 7. Red arrows are drawn beneath genes expressed from maternal alleles in at least some tissues; green arrows indicate genes expressed from paternal alleles. Yellow shading indicates ICRs 5' of *H19* (top left) and the *KCNQ1OT1/Kcnqlot1* coding region within the *KCNQ1/Kcnql* gene. Deletion of the ICR 5' of *H19* causes biallelic expression of *H19* when the deletion is inherited from the male parent (Thorvaldsen *et al.* 1998). Paternal transmission of a deletion of part of the CpG island associated with the 5' end of the *Kcnqlot1* gene caused maternal expression of those local genes that are normally expressed from paternal alleles (Fitzpatrick *et al.* 2002). BWS in humans can result from loss of imprinting of either the sub-domain associated with *H19-IGF2* or that associated with *KCNQ1OT1*. Mouse genomic sequences are shown. ESTs, expressed sequence tags. Human cons is a measure of homologous mouse and human genomic sequence.

Figure 2. Sex-specific exons in the *Dnmt1* gene. Genomic imprints are maintained in preimplantation embryos by Dnmt1o, the product of a *Dnmt1* mRNA that contains the oocyte-specific promoter and first exon. Dnmt1o lacks the 118 N-terminal amino acids found in the somatic form of Dnmt1 but has full activity; the deleted amino acids allow the protein to accumulate to high levels in growing oocytes by uncoupling protein stability from the cell cycle (Ding & Chaillat 2002).

Figure 3. Mitotic segregation of imprinted state after a failure of maintenance methylation during preimplantation development. This mechanism is proposed to explain the relatively high incidence of BWS in monozygotic twins and the high frequency of discordance in such twin pairs.

dictyate oocytes, which are arrested late in the prophase of meiosis I and which will not undergo a mitotic division prior to fertilization, whereas paternal imprints are established in the perinatal prospermatogonia, which will undergo many mitotic divisions as spermatogonia prior to the beginning of meiosis (Lucifero *et al.* 2002). In the case of the two imprinted sub-domains implicated in the etiology of BWS, the ICR 5' of *H19* undergoes *de novo* methylation in male germ cells at a very early stage of gametogenesis, while the ICR associated with the promoter of the *KCNQ1OT1* gene undergoes *de novo* methylation at a relatively late stage of female gametogenesis. The imprinted expression of both sub-domains depends on allele-specific methylation as shown by the fact that global genome demethylation in mice homozygous for loss of function alleles of the DNA methyltransferase-1 (*Dnmt1*) gene activates the paternal allele of *H19*, with inactivation of the paternal *IGF2* gene, while the maternal allele of *Kcnq1ot1* is activated and *Kcnq1* and other genes in the associated imprinted sub-domain lose allele-specific expression (Li *et al.* 1993; Caspary *et al.* 1998). When the imprinting factor Dnmt3L is removed from oocytes, oogenesis is normal but the heterozygous offspring of homozygous females die at 9.5 days *post coitum* with biallelic expression of genes in the imprinted sub-domain associated with the *Kcnq1ot1* ICR (Bourc'his *et al.* 2001).

Such embryos show normal monoallelic expression of the *H19* and *Igf2* genes, which is consistent with the paternal origin of the *H19* imprint and the usual reciprocal pattern of expression of *H19* and *Igf2*.

It has been reported that a loss of imprinting of either the *H19-IGF2* or *KCNQ1OT1* sub-domains can occur independently to yield clinically indistinguishable forms of BWS, although Wilms' tumour is reported to be more common when *H19-IGF2* is involved (DeBaun *et al.* 2002). It is not immediately clear how deregulation of two sets of genes with no apparent overlap in function can give rise to very similar syndromes. However, paternal transmission of deletions of *Igf2* (DeChiara *et al.* 1991) or the promoter region of *Kcnq1ot1* (Fitzpatrick *et al.* 2002) both yield mice of grossly normal phenotype except for a *ca.* 25% reduction in size. It is possible that the phenotypes are not directly due to the effects of the deleted sequences but to an unidentified gene (or genes) whose regulation depends on the normal imprinted expression of both the *H19-Igf2* and *Kcnq1ot1* sub-domains. The product of such a gene could act *in trans* and need not necessarily be linked to *H19-Igf2* or *Kcnq1*. The similar phenotypes of *Igf2* and *Kcnq1ot1* mutants (DeChiara *et al.* 1991; Fitzpatrick *et al.* 2002), and of BWS patients with loss of imprinting at either *H19-IGF2* or *KCNQ1OT1* (DeBaun *et al.* 2002), are otherwise difficult to explain.

2. THE DNMT1O ERROR MODEL FOR BWS DISCORDANCY IN MONOZYGOTIC TWINS

Monozygotic twins are normally of identical genotype. Why should there be so many monozygotic twins with BWS, why are such twins nearly always discordant for BWS, and why are such twins predominantly female (reviewed by Junien 1992)? Molecular genetic studies of a variant of Dnmt1 may provide an answer to these strikingly non-Mendelian questions.

The most abundant form of DNA methyltransferase in vertebrates is Dnmt1 (Bestor *et al.* 1988); a close homologue is present in flowering plants and deuterostomes, but has not yet been found in protostomes (reviewed by Bestor 2000). The Dnmt1 protein of mouse and human catalyses methyl transfer to hemimethylated DNA substrates at a rate that is 7–30-fold higher than the rate of methylation of the corresponding DNA in the unmethylated state. As a result of this biochemical finding Dnmt1 has been assigned a role in the maintenance of genomic methylation patterns during the S phase rather than a role in their establishment. It should be borne in mind that the only detectable *de novo* DNA methyltransferase activity in lysates of vertebrate cells is Dnmt1, which is abundant and has only a preference, not a requirement, for hemimethylated substrates. Recent data suggest that Dnmt1 can mediate some *de novo* methylation *in vivo* in the absence of Dnmt3A and Dnmt3B (Lorincz *et al.* 2002), which were previously believed to be the sole *de novo* DNA methyltransferases of mammals.

The *Dnmt1* gene is unusual in that it bears sex-specific promoters and 5' exons (Mertineit *et al.* 1998; figure 2). A male-specific first exon introduces multiple short open reading frames upstream of the Dnmt1 initiation codon and would be expected to prevent translation of the mRNA; this exon (termed exon 1p) is present in the Dnmt1 mRNA only in pachytene spermatocytes, which lack detectable Dnmt1 protein. Growing oocytes (in which imprints are established) contain a second Dnmt1 mRNA in which an alternative 5' exon causes translation to be initiated at what is, normally, the second ATG codon; the protein product lacks 118 N-terminal amino acids relative to the full-length protein, which is present in dividing somatic cells of both sexes. The oocyte-specific Dnmt1 mRNA has been seen only in oocytes and is termed Dnmt1o. This protein has a substantially increased half-life relative to full-length Dnmt1.

Dnmt1o is the only form of Dnmt1 protein present in normal mouse oocytes and preimplantation embryos (Mertineit *et al.* 1998; Howell *et al.* 2001). Full-length Dnmt1 is exclusively nuclear in all somatic cells, but Dnmt1o protein is cytoplasmic in the mature oocyte and in embryos of the 2-cell, 4-cell, 16-cell, morula and blastocyst stages; it is nuclear only at the 8-cell stage (Carlson *et al.* 1992; Howell *et al.* 2001). Full-length Dnmt1 protein is not produced until implantation. Dnmt1o is functionally equivalent to full-length Dnmt1; the function of the N-terminal 118 amino acids missing from Dnmt1o involves coupling of protein stability to the cell cycle (Ding & Chaillet 2002). Loss of these sequences is likely to allow Dnmt1o to accumulate to high levels in non-dividing growing oocytes.

A mechanism whereby failure of Dnmt1o to maintain a methylation imprint at *KCNQ1OT1* at one preimplantation S phase is shown in figure 3, in which it is proposed that asymmetry is introduced by a failure of maintenance methylation during a single cell cycle at, or just prior to, the twinning event. The resulting hemimethylated daughter duplex would be converted, in the next S phase, to one fully methylated sister chromatid and an unmethylated sister chromatid, which would segregate to different blastomeres. This introduces functional asymmetry to sister chromatids that are normally identical. These would then separate in the twinning event; the relatively high frequency of monozygotic twins with BWS could reflect increased twinning rates owing to growth asymmetry created by increased rates of expression of growth-enhancing genes in the population of cells that had lost imprinting at *KCNQ1OT1*. Early twinning could give rise to monozygotic twins with complete discordance; mosaicism manifested as hemihypertrophy could result from development in mixed early embryonic cell populations, in which some cells had lost imprinting at *KCNQ1OT1* and others had retained normal imprinting. This model is supported by studies in which exon 1o was deleted via homologous recombination and Cre-mediated excision (Howell *et al.* 2001). This mutation removed all Dnmt1o protein from the oocyte. It was found that removal of Dnmt1o caused one half of the normally imprinted alleles to be demethylated, and a loss of imprinting phenotype consistent with mosaic reactivation of normally silenced imprinted alleles was observed. We suggest that maintenance methylation at the DMR of *KCNQ1OT1* is less efficient than at other DMRs, and that BWS arises when maintenance methylation of *KCNQ1OT1* fails to occur. *Dnmt0* or *Dnmt1* is also required for X inactivation, and association of *DNMT1* with one of the X chromosomes in females may reduce the amount available for maintenance methylation of *KCNQ1OT1*, thereby accounting for the sex bias.

The loss of imprinting at *KCNQ1OT1* might not be purely stochastic; it might involve unidentified paternally expressed genes that demethylate and reactivate maternally repressed growth-enhancing genes. This model, which invokes the intragenomic conflict model of Haig and colleagues, stems from competition for maternal resources among offspring sired by different males, either within or between litters. A male that evolves a trait that causes its offspring to grow faster *in utero* will enjoy a selective advantage if the larger size increases the probability of survival to breeding age. As females are related to an equal extent to all their offspring, while a male is related only to those offspring that it sires, Haig has proposed that maternal imprinting arose to equalize maternal resources among all offspring, while paternal imprinting arose to allow offspring sired by one male to grow to larger size at the expense of offspring sired by other males (Haig & Graham 1991). As the loss of expression of paternal *KCNQ1OT1* mainly affects embryo size (Fitzpatrick *et al.* 2002), reactivation of the silent maternal allele, in combination with the active paternal allele, would be expected to increase embryo size. This reasoning suggests that it would therefore be in the interest of the male to evolve a new function that demethylated and reactivated maternal *KCNQ1OT1*. This may, in turn, answer a long-standing question in the DNA methylation field: why is there so

much Dnmt1o in the oocyte? Measurements suggest that there is 1000–40 000-fold more Dnmt1 protein in the oocyte than is required for maintenance methylation during the S phase of a somatic cell (Carlson *et al.* 1992), and removal of all Dnmt1o affects the methylation status only at imprinted loci with little effect on global methylation levels (Howell *et al.* 2001). It is suggested that there exists a paternally expressed imprinted gene that has evolved to relieve maternal silencing by opposing maintenance methylation mediated by Dnmt1o during preimplantation development, and that this gene placed the female under selective pressure to evolve a means to counteract this factor. The response to this paternal methylation inhibitor may have been the accumulation of very large maternal stores of Dnmt1o protein, that overwhelm the action of the paternal inhibitor at methylated imprinted loci, and the cases of BWS that are due to loss of imprinting at KCNQ1OT1 may be a manifestation of this sexual conflict.

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GLOSSARY

BWS: Beckwith–Wiedemann syndrome
ICR: imprinting control region