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Igf2 imprinting in development and disease

WOLF REIK[1]*, MIGUEL CONSTANCIA[1], WENDY DEAN[1], KAREN DAVIES[1], LUCY BOWDEN[1], ADELE MURRELL[1], ROBERT FEIL[1], JOERN WALTER[2] and GAVIN KELSEY[1]

[1]Laboratory of Developmental Genetics and Imprinting, Developmental Genetics Programme, The Babraham Institute, Cambridge, United Kingdom and [2]Max-Planck Institute of Molecular Genetics, Berlin, Germany

ABSTRACT ***Igf2*** **is one of the first imprinted genes discovered and occupies a centre stage in the study of imprinting. This is because it has dramatic effects on the control of fetal growth, it is involved in growth disorders and in cancer, it interacts with products of other imprinted genes, and its imprinting status is under complex regulation in a cluster of tightly linked imprinted genes. Here we review briefly the key features of** ***Igf2*** **imprinting in normal development and in disease, and hope to show what a fascinating subject of study this gene and its biology provides.**

KEY WORDS: *imprinting, DNA methylation, fetal growth, mouse development, genetic disease*

Role of *Igf2* in placenta and fetus

The mouse (and other mammalian) *Igf2* gene encodes a single polypeptide (Fig. 1). This polypeptide is thought to have autocrine (same cell), paracrine (surrounding cells), and endocrine (circulation) actions (Efstratiadis, 1998; see below). The IGF-II ligand signals through the IGF1 receptor and the Insulin receptor, and is also bound by the IGF2 receptor (which does not transduce any signal). The biological action of IGF-II is further modulated by IGF binding proteins in serum and tissues. Various effects of IGF-II *in vitro* on cell proliferation and apoptosis are documented, however, its precise roles in growth *in vivo* are not fully established (Efstratiadis, 1998). A recent analysis suggests effects on both dry weight and wet weight, but less on DNA content, suggesting roles in cell and tissue reorganisation as well as cell proliferation (Gardner *et al.*, 1999).

Igf2 transcripts are primarily produced in mesodermal, endodermal and extraembryonic tissues, and different enhancers are largely responsible for this tissue specificity (see below). The three promoters P1–P3 are transcribed in all these tissues, whereas promoter PO is active specifically in the placenta (Moore *et al.*, 1997). Although there is some potential for translational differences between these transcripts, they are all thought to be translated (Nielsen *et al.*, 1999). While *Igf2* begins to be transcribed shortly after implantation first in extraembryonic tissues, and then throughout mesodermal and endodermal tissues in postimplantation embryos (Lee *et al.,* 1990), the action of gene expression on growth is only seen from E13 onward (Efstratiadis, 1998). Since this is also the case for some of the other components of the IGF/INS pathway (IGF-1, IGF1R), it is likely that critical components of the signalling pathways are only assembled after this point. Both under and overexpression of *Igf2* has dramatic and apparently dose dependent effects. Fetuses (and their placentae) completely lacking IGF-II are 40% growth retarded at birth (60% of normal size) and are apparently normally proportioned dwarfs (De Chiara *et al.*, 1990). Fetuses lacking *Igf2* expression in endoderm (liver, gut, etc.) through knockout of the endoderm specific enhancers located downstream of *H19* (Fig. 3) are 80% of normal size at birth and are also apparently normally proportioned, which may attest to the importance of endocrine IGF-II action (Leighton *et al.*, 1995a). The endoderm enhancer KO mice also show that lowered *Igf2* expression limited to the fetus (with normal levels in the placenta) has an effect independent of the placenta (Leighton *et al.,* 1995a). Overexpression of *Igf2* can increase size at birth up to 160% (Sun *et al.,* 1997), and size at E17 up to 200% (Eggenschwiler *et al.,* 1997), in a manner dependent on IGF-II dosage. High levels of overexpression also lead to various malformations and intrauterine death (Eggenschwiler *et al.*, 1997; Sun *et al.,* 1997; see below). Individual organs can be enlarged in proportion to their *Igf2* levels, suggesting autocrine or paracrine control (Sun *et al.*, 1997). The issue of paracrine or endocrine control is not fully resolved, but new genetic models including the endoderm enhancer KO and a mutant that we are studying in collaboration with B. Cattanach (Harwell) which lacks *Igf2* expression in mesoderm (K. Davies *et al.*, unpublished), may allow further clarification.

While IGF-II action can affect the growth of the fetus (through signalling via IGF1R and INSR), it has a particularly unique and important action in the placenta. Thus, while *Igf1* and *Igf1r* KOs result in small fetuses and normal size placentae, all manipula-

***Address for reprints:** Laboratory of Developmental Genetics and Imprinting, Developmental Genetics Programme, The Babraham Institute, Cambridge CB2 4AT, United Kingdom. FAX: 44 (0)1223 496030. e-mail: wolf.reik@bbsrc.ac.uk

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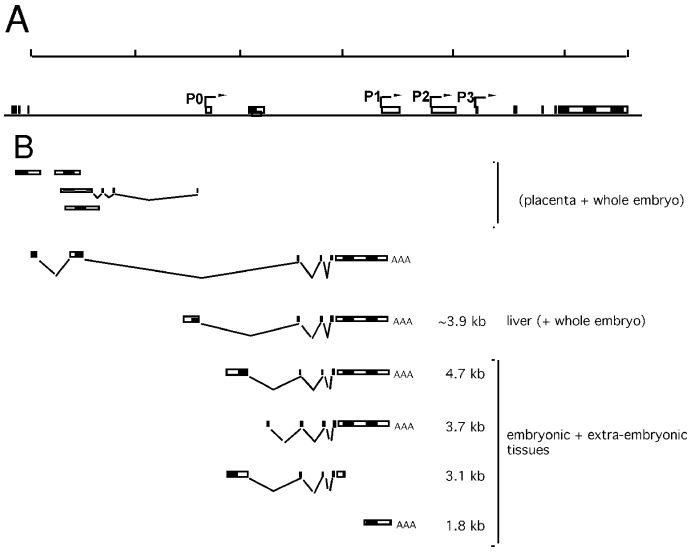

Fig. 1. The mouse *Igf2* gene. (A) *Genomic map of the mouse Igf2 gene. The Igf2 gene maps to distal chromosome 7 and is flanked 5' by the insulin II gene (Ins2). Multiple transcripts are produced from alternative promoters (horizontal arrows): P0, P1, P2 and P3. Igf2 exons (filled boxes): U1, U2, E1-E6. The coding sequences are located in exons 4-6 and are shown as open boxes. A CG rich repeat is found in the U2 exon.* **(B)** *Igf2 transcripts. The various Igf2 transcripts are shown below the structure of the Igf2 gene. Extensive antisense transcription occurs through the Igf2 upstream region (Igf2as-a,b,c). The function of these untranslated transcripts remains unknown. All different spliced sense transcripts contain exons 4-6 that code for the 67 amino acid IGF-II protein. The P0 transcript is placenta-specific. P1 transcripts are expressed in fetal and neonatal liver, predominantly. P3 and P4 transcripts are the major embryonic and fetal promoters. The 3' UTR sequences vary between transcripts because of alternative polyadenylation (AAA).*

tions involving *Igf2* or *Igf2r* result in altered growth of the placenta (Efstratiadis, 1998). IGF-II is thought to signal through a separate receptor in the placenta which remains unknown (Efstratiadis, 1998). Therefore, placental IGF-II action and its effect on the fetus could thus far not be examined specifically. However, since a placenta specific *Igf2* transcript has been recently identified (Moore *et al*., 1997), which is transcribed in the labyrinthine trophoblast where maternal and fetal circulation interface (M. Constancia *et al*., unpublished), the question can now be addressed. Significantly, a knockout of the placenta specific transcript results in smaller size of the placenta and fetus from E14, and fetal weight is 70% of normal at birth (M. Constancia *et al*., unpublished). Therefore, reduction of size of the placenta or dysfunction of the labyrinthine trophoblast leads to severe intrauterine growth retardation.

The importance of placental IGF-II production is confirmed in another system, in which chimeric conceptuses were constructed that lacked IGF-II in trophectoderm and hence labyrinthine and spongiotrophoblast of the placenta, but the fetus was normal genotype (Gardner *et al*., 1999). The effects on fetal and placental weights on E16.5 were identical to the ones recorded in the placental specific KO (M. Constancia *et al.,* unpublished; Gardner *et al*., 1999).

The observation that genes for fetal growth factors which are particularly important for placental growth and development are imprinted *(Igf2, Igf2r),* whereas those whose roles are limited to the fetus are not *(Igf1, Igf1r, Insr)*, raises the question of whether the primary selection for imprinting comes from action of growth factors in the placenta with a direct effect on nutrient transfer from mother to offspring. It also raises the possibility that there might be differences in mechanistic aspects of imprinting in placenta and fetus.

IGF-II interacts with the products of other imprinted genes (*Igf2r, H19,* probably *Grb10,* Miyoshi *et al*., 1998) and it will be interesting to see whether other imprinted growth related genes also interact with the IGF pathway, in particular perhaps in the placenta.

Mechanisms of *Igf2* imprinting

The *Igf2* gene is part of a cluster of imprinted genes on distal chromosome 7 in the mouse, with a homologous cluster on the syntenic region on chromosome 11p15.5 in the human (Paulsen *et al*., 1998; Fig. 2). The *Igf2* gene is paternally expressed (De Chiara *et al.,* 1991). A number of sequences that are important for *Igf2* imprinting and expression have now been defined. *Igf2* imprinting is under regional control particularly influenced by the neighbouring maternally expressed *H19* gene (Leighton *et al*., 1995b; Ainscough *et al*., 1997; Brannan and Bartolomei, 1999). The influence of the *H19* control sequences apparently also extends to *Ins2*, but probably not further to other genes in the cluster (Caspary *et al*., 1998). Whether other genes or control sequences in the cluster have an influence on *Igf2* or *H19* imprinting or expression is currently uncertain.

Two sequences have been identified so far with clearly defined roles *in vivo* in imprinting and expression of *Igf2/H19*. The first is a set of enhancers called the endoderm enhancers which are located 3' of the *H19* gene (Fig. 3). Knockout of these enhancers abolishes expression of *H19* (on the maternal chromosome) in mainly endodermal tissues such as liver and gut, and of *Igf2* (on the paternal chromosome) in the same tissues (Leighton *et al*., 1995a). This establishes that these enhancers are shared by both genes. So, how is the action of the enhancers limited to either gene on different parental chromosomes? Upstream of *H19* is located a differentially methylated region (DMR) with paternal methylation and maternal undermethylation (the paternal methy-

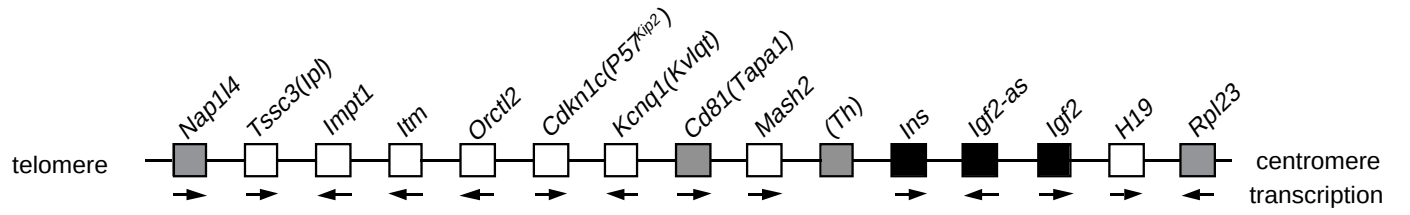

Fig. 2. **Imprinting cluster on distal chromosome 7.** *Schematic physical map of the distal chromosome 7 imprinting cluster in mouse showing imprinting status and transcriptional orientation. Maternally expressed genes are shown in red, paternally expressed genes in blue, non-imprinted genes in black and genes for which the imprinting status is unknown in grey. Th has so far not been mapped in mouse, hence its position (based on human data) is shown in parentheses. For details see http://www.mgu.har.mrc.ac.uk/imprinting/implink.html.*

lation also extends into the *H19* promoter and gene body and is thought to silence the paternal gene copy). When this region is deleted, *H19* is no longer methylated on the paternal chromosome, and is now expressed. Similarly, the maternal copy of *Igf2* is expressed from a chromosome with the deletion (Thorvaldsen *et al*., 1998). The currently favoured interpretation is that this DMR represents some sort of chromatin insulator or boundary element which when methylated allows access of the *Igf2* promoters to the *H19* enhancer (Thorvaldsen *et al*., 1998; Webber *et al*., 1998). In its unmethylated state, the element represents a closed boundary which prevents activation of the *Igf2* promoters by their enhancers. Interestingly, a specialised non-histone type chromatin structure has recently been shown to exist on the maternal (unmethylated) DMR region (Hark and Tilghman, 1998; Szabo *et al*., 1998; Khosla *et al*., 1999). The DMR consists of various elements, including a direct repeat structure and a potential silencer (Lyko *et al*., 1997), whose detailed functions *in vivo* are unclear at present. The knockout of the DMR also results in a reduced level of *Igf2* expression on a chromosome that also expresses *H19* (and vice versa) suggesting that the promoters of both genes also compete for the shared enhancers (Thorvaldsen *et al*., 1998). As mentioned earlier, enhancers for lineages other than endoderm have so far not been found, but we are studying a radiation induced mutant in which *Igf2* expression is abolished in mesodermal tissues (K. Davies *et al*., unpublished).

How the *Igf2* promoters access the distant enhancers (or are prevented from doing so presumably by an unmethylated DMR) is unclear. It is remarkable that strong DNase I hypersensitive sites are present in *Igf2* promoters and upstream on both parental chromosomes (Sasaki *et al*., 1992; Feil *et al*., 1995). The *Igf2* gene itself also has three DMRs (Fig. 3) but whether these have functions independent of *H19* and its DMR/enhancers or in conjunction with these is currently not known (Sasaki *et al*., 1992; Feil *et al*., 1994; Moore *et al*., 1997). Knockouts in DMR1 and DMR2 have been prepared, however, and are under study, so that some of these answers should soon emerge.

The *H19* DMR seems to carry a 'germline methylation imprint' in the sense that the sperm copy is methylated, the oocyte copy is not, and these methylation patterns are inherited through all stages of development (Olek and Walter, 1997; Tremblay *et al*., 1997), except in germ cells where they are switched as appropriate. DMR1 in *Igf2* (which was the first DMR to be discovered, Sasaki *et al*., 1992) is apparently not differentially methylated in germ cells but becomes so soon after fertilisation (Shemer *et al*., 1996). DMR2 is differentially methylated in germ cells, and loses this methylation in the early preimplantation embryo, which then becomes re-established later on (Oswald *et al*., unpublished). Thus, whether these sequences carry germline imprints is not totally clear, however, it should be borne in mind that there could be epigenetic memory systems other than methylation that interact with the methylation system (Pickard *et al*., unpublished). The methylation patterns at *Igf2* DMRs 0-2 are not independent of the *H19* DMR/gene, but whether this is because of altered expression of *Igf2* as a consequence of deletion of the *H19* DMR/gene, or whether there are elements that are responsible for regional establishment and spreading of the 'epigenotype' is currently not clear (Forné *et al*., 1997; Moore *et al*., 1997).

Although other genes in the cluster also have DMRs, and the cluster as a whole displays asynchronous DNA replication during the cell cycle as a global feature (Kitsberg *et al*., 1993), other regional or local control elements have so far not been defined (but

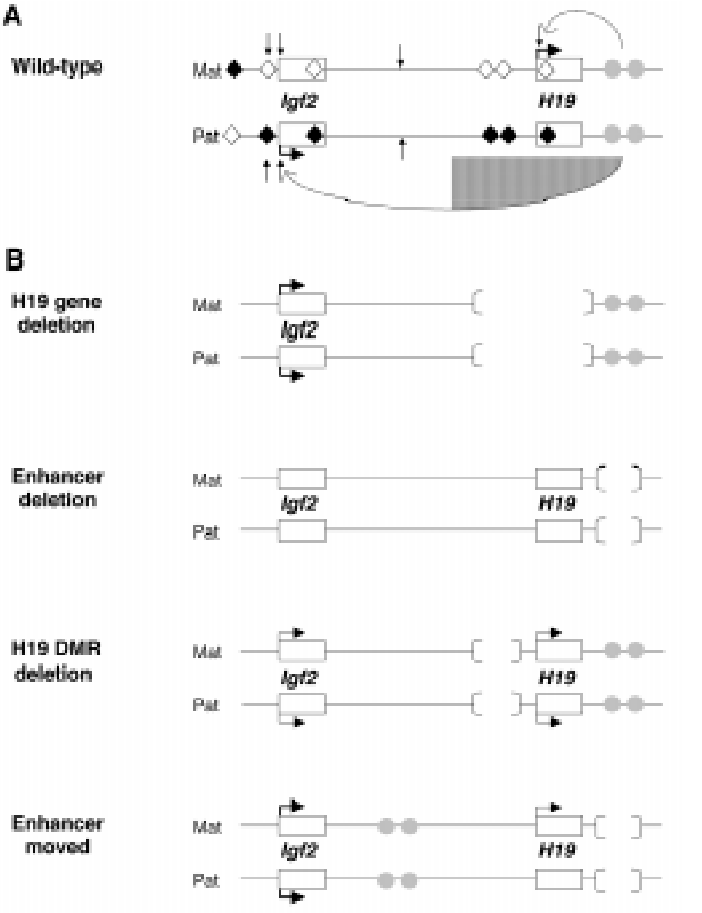

Fig. 3. **Epigenetic modifications in the *Igf2-H19* region and their functional testing**. **(A)** *Wildtype situation on both maternal and paternal chromosomes. Open diamonds, unmethylated DMRs, filled diamonds, methylated DMRs. Igf2 has DMRs 0-2, H19 has an upstream DMR which may be a boundary element, and the promoter and gene is also differentially methylated. Arrows, DNase I hypersensitive sites in chromatin. Circles, endoderm specific enhancers.* **(B)** *Knockout experiments that address the function of some of the elements. Knockout of the H19 gene and its upstream region results in biallelic expression of Igf2. Deletion of the endoderm enhancers results in reduced expression of H19 and Igf2 in endodermal tissues, thus showing that the enhancers are used by both genes. Deletion of the H19 DMR results in biallelic expression of both genes, suggesting a boundary element has been removed. Moving the enhancers results in biallelic expression of Igf2 but H19 is only expressed from the unmethylated allele. Other regions have not yet been tested.*

see below for some hints). In any event, the *H19–Igf2* system of genes is an excellent model system for the study of germline imprints and regional control of imprinting. The mechanistic details of this system are under intense scrutiny and discussion (Constancia *et al*., 1998; Reik and Walter, 1998; Surani 1998; Tilghman 1999; Brannan and Bartolomei, 1999), but presentation of these details goes beyond the scope of the current review.

Altered IGF2 expression in disease

There are now a number of disease situations in which levels of *IGF2* expression are altered (Ward 1997; Morison and Reeve, 1998; Feinberg 1999). *IGF2* expression is often increased and this

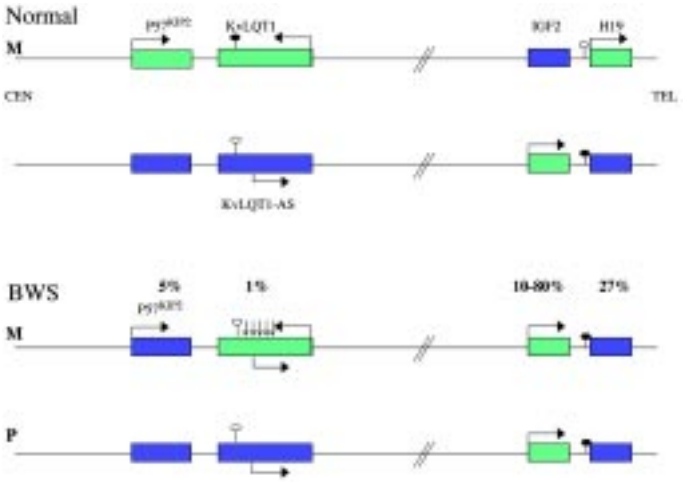

Fig. 4. Altered imprinting of chr11p15.5 is involved in Beckwith-Wiedemann syndrome. *Mutations and epimutations in the p57^{KIP2}, KvLQT1, IGF2 and H19 genes on chromosome 11p15.5 have so far been shown to be involved in the aetiology of BWS. Light boxes, expressed genes; dark boxes, silent genes; X, point mutations; arrows in KvLQT1, breakpoint cluster for translocations; filled/open circles, methylated/demethylated CpGs; arrows above the boxes, direction of sense transcription; arrows below boxes, antisense transcription. The frequency of genetic and epigenetic mutations in BWS patients is also indicated. These are very approximate. The frequency of LOI of IGF2 varies between studies [(e.g., compare Reik and Maher (1997) and Lee et al., (1999)].*

is associated with enhanced growth or increased cell proliferation. Most frequently increases are caused by loss of imprinting (LOI), i.e., expression of the otherwise repressed maternal allele in addition to the paternal one. The *IGF2* gene is part of a cluster of imprinted genes on distal chromosome 7 (in mouse) and 11p15.5 (in human) as discussed (Fig. 2), and imprinting mechanisms are thought to be regional, so potentially a variety of mechanisms could lead to altered imprinting/expression of *IGF2* (Reik and Maher, 1997; Feinberg 1999).

The dependence of *Igf2* imprinting on *H19* has already been described. Further telomeric (Fig. 2), lies the *Mash2* gene which has an important role in placenta development, where imprinting controls have not been precisely defined. Remarkably though, it is possible that imprinting of *Mash2* can proceed independently of DNA methylation, which would further support the notion of epigenetic memory other than methylation (Caspary *et al.*, 1998). Close to *Mash2* there is a region of transcripts including *Tapa1* (*Cd81*) which may show some bias in gene expression towards one of the parental alleles, but may not show clear cut imprinting as do other genes in the cluster (Caspary *et al.*, 1998). Further centromeric are *Kvlqt1/Kcnq1* (potassium channel) *p57^{Kip2}/Cdkn1c* (negative cell cycle regulator), *Orctl2* (organic cation transporter), *Hm* (transmembrane domain) *Impt1* (transporter-like), and *Tssc3/Ipl,* all bona fide imprinted genes with maternal expression. *Kvlqt1/Kcnq1* is overlapped by an antisense transcript with paternal expression (Lee *et al.*, 1999; Smilinich *et al.*, 1999), and this system may therefore be in 'expression competition' as are *H19-Igf2* (Brannan and Bartolomei, 1999), *Igf2r–Igf2r*-antisense (Wutz *et al.*, 1997) and possibly others. Although various DMRs have been described in these genes, the controls have not been elucidated in functional assays.

Loss of imprinting of *Igf2* has been described as an isolated somatic event, largely associated with various pediatric and adult cancers, or an embryonic or germline event associated with the overgrowth and cancer syndrome Beckwith-Wiedemann syndrome (see below). Many cancers (but with notable exceptions) show LOI of *IGF2* (Feinberg, 1999). Such LOI could either be causally involved in tumour initiation or growth, or be an epi-phenomenon because of altered maintenance of DNA methylation and potentially imprinting in tumours. To our knowledge, a large scale analysis of the stability or otherwise of imprints in tumours has not yet been carried out. However, there is evidence that overexpression of *IGF2* can indeed lead to tumour initiation or progression (Christofori *et al.*, 1994; Bates *et al.*, 1995).

In terms of mechanisms, the best studied system is Wilms' tumour, a childhood tumour of the kidney. A large proportion of tumours with *IGF2* LOI also have maternal methylation of the *H19* gene (Moulton *et al.*, 1994; Steenman *et al.*, 1994). This is consistent with the model described above in which methylation of the *H19* DMR would allow access of *IGF2* promoters to the shared enhancers. This hypermethylation of *H19* can sometimes be observed in adjacent healthy tissues, suggesting that it is an early, or even predisposing event, in tumourigenesis (Moulton *et al.*, 1994; Okamotomo *et al.*, 1997). However, no mutational mechanisms leading to this epigenetic switch have been identified, and to our knowledge, this type of event has not been observed in rare familial cases of the disease. Hence, it is conceivable that this epigenetic switch could be the result of 'epimutation'. Although this could arise randomly, a proposal has been made for a mechanism by 'methylation transfer' from the methylated paternal to the unmethylated maternal *H19* DMR (Bestor and Tycko, 1996). Confirmation of this mechanism awaits analysis in a genetic model.

Germline or early embryonic alterations in *IGF2* imprinting could lead to LOI in many cells of the developing organism (Dean *et al.*, 1998). This seems to be the case in the fetal overgrowth syndrome, Beckwith-Wiedemann syndrome, in which the majority of patients show apparent LOI at least in fibroblasts cultured from their skin (Weksberg *et al.*, 1993; Reik *et al.*, 1995; Reik and Maher, 1997). In mouse models, the majority of the symptoms of the disease can indeed be attributed to overexpression of *Igf2* (Sun *et al.,* 1997; Eggenschwiler *et al.,* 1997*)*. The mechanisms by which LOI arises, and the molecular pathogenesis in BWS patients in which there is no LOI of *IGF2*, are apparently complex but all molecular abnormalities detected so far are linked to 11p15.5 (Fig. 4; Reik and Maher, 1997). The majority of BWS patients are sporadic, and familial cases are rare. A number of sporadic cases show maternal methylation of *H19*, with associated LOI of *IGF2* (Fig. 4; Reik *et al.*, 1995). Surprisingly, other patients with LOI of *IGF2* show normal imprinting of *H19* (Joyce *et al.*, 1997). A minority of BWS patients have maternally inherited translocations in 11p15.5, with several breakpoints in *KvLQT1* (Lee *et al.*, 1997a), or further centromeric outside of the present clusters (Mannens *et al.*, 1996). In two families in which this could be analysed, translocation was associated with LOI of *IGF2* (Brown *et al.*, 1996; Smilinich *et al.*, 1999), but the mechanism of this is unknown. In another series of sporadic patients, LOI of the *KvLQT1* antisense gene occurred at a high frequency (Fig. 4; Lee *et al.*, 1999; Smilinich *et al.*, 1999) which in some cases was associated with LOI of *IGF2* but in others not. Hence, currently it

is unclear whether mutational (translocation) or likely epigenetic (LOI of antisense gene) events in the *KvLQT1* region interfere with *IGF2* imprinting/expression. If they do not, this would reinforce the notion of a bipartite structure of the cluster, with potentially independent imprinting controls (Caspary *et al*., 1998). However, strikingly, it would also lend support to the previous suggestion that alterations in linked imprinted genes in the cluster could result in similar phenotypes, perhaps because these genes interact in common physiological pathways (Reik and Maher, 1997). Indeed, the only point mutations discovered so far in BWS patients have been found in the cell cycle inhibitor *p57^{KIP2}/CDKN1C* (Fig. 4; Hatada *et al*., 1996; Lee *et al*., 1997b; O'Keefe *et al*., 1997; Lam *et al*., 1999), and these do not seem to be associated with LOI of *IGF2*. These mutations are more frequent in familial cases than in sporadic ones (Lam *et al*., 1999), whereas the epigenetic alterations in *H19* or *KvLQT1* antisense are exclusive to sporadic patients. The suspicion is therefore that the majority of BWS patients have epimutations –in either *H19* or *KvLQT1* antisense– rather than genetic mutations.

Conclusions

The study of imprinting and phenotypic effects of the *Igf2* gene, its imprinted neighbours in the cluster, and its interacting components encoded elsewhere in the genome, will continue to be a fascinating subject of imprinting research. Because growth and cell proliferation are such central themes in imprinting, progress in understanding the regulation of *Igf2* will continue to lead to key insights into the biology and pathology of growth and cell proliferation.

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