

Epigenetic regulation of the *Igf2/H19* gene cluster

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Abstract

Igf2 (insulin-like growth factor 2) and *H19* genes are imprinted in mammals; they are expressed unevenly from the two parental alleles. *Igf2* is a growth factor expressed in most normal tissues, solely from the paternal allele. *H19* gene is transcribed (but not translated to a protein) from the maternal allele. *Igf2* protein is a growth factor particularly important during pregnancy, where it promotes both foetal and placental growth and also nutrient transfer from mother to offspring *via* the placenta. This article reviews epigenetic regulation of the *Igf2/H19* gene-cluster that leads to parent-specific expression, with current models including parental allele-specific DNA methylation and chromatin modifications, DNA-binding of insulator proteins (CTCFs) and three-dimensional partitioning of DNA in the nucleus. It is emphasized that key genomic features are conserved among mammals and have been functionally tested in mouse. ‘The enhancer competition model’, ‘the boundary model’ and ‘the chromatin-loop model’ are three models based on differential methylation as the epigenetic mark responsible for the imprinted expression pattern. Pathways are discussed that can account for allelic methylation differences; there is a recent study that contradicts the previously accepted fact that biallelic expression is accompanied with loss of differential methylation pattern.

Introduction

The aim of this article has been to review and discuss the imprinted gene complex *Igf2/H19*. An imprinted

gene differs from non-imprinted counterparts since it is not expressed equally from each of the two parental alleles. Instead, one allele is preferentially expressed, some imprinted genes being expressed from the paternally inherited allele, such as *Igf2* and *Dlk-1*, and others characteristically expressed from the maternally inherited allele, such as *H19*, *Igf2r* and *Snrpn*.

In the 1970s, a theory appeared that proposed complex interplay of different interests between parents and offspring within the endosperm of flowering plants, a tissue analogous to the mammalian placenta. The theory postulated that offspring, especially when derived from different fathers, compete with each other for maternal resources, while the mother attempts to provide resources equally to all offspring since they are equally related to her (1,2). A decade later, the discovery that for correct development a mammalian one-cell embryo needs one pro-nucleus from a parent of each sex made it clear that there is functional distinction between paternal and maternal chromosomes. The zygote cannot function with two maternal or two paternal sets of chromosomes (3). This was contradictory to the Mendelian law of inheritance which assumes maternal and paternal gene copies are essentially equivalent.

Haig and Westoby (4) summarized these ideas concerning conflict of different interests in mammals, marsupials and flowering plants and presented a model describing how evolution could favour an allele that, when derived from the paternal genome, promotes acquisition of maternal resources. This is now widely accepted as a likely explanation for evolution of imprinted gene expression, at least at some loci, and is known as the parent–offspring conflict theory (or conflict theory). They also hypothesized that evolutionary response in the maternal allele would be to use silencing in an attempt to forestall foetal overgrowth. It is applicable to mammals and flowering plants since both start life as dependent on their mother for sustenance.

Insulin-like growth factor 2 protein (*Igf2*) was well characterized and known to induce cell proliferation

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in vitro (5,6). DeChiara *et al.* (7,8) studied mice with *Igf2* gene deletions and found that if the gene deletion was inherited *via* the egg, the offspring was phenotypically normal, but when it came *via* the sperm offspring were growth deficient with birth weight approximately only 60% of a normal mouse. Paternal expression of *Igf2* was demonstrated in the majority of tissues where the gene is active and it was thus the first imprinted gene to be identified (8). Paternal expression of a growth-promoting gene also validated the genetic conflict theory. Remarkably, this finding was soon followed by the discovery of *Igf2r*, a gene expressed from the maternally inherited allele with a growth-inhibitory function, which was again consistent with the conflict theory. Disruption of the maternal copy of Igf type 2 receptor (*Igf2r*) gene in mice was associated with foetal overgrowth (9). Since then, many more imprinted genes have been identified and in mouse over 100 are currently known (<http://www.mousebook.org/catalog.php?catalog=imprinting>). Many imprinted genes have roles in foetal growth and development, but there are also those with functions in energy homeostasis (11,12) and also brain function and behaviour (13). Whether all of these genes fit with the parental conflict theory has been a matter of debate, as described in (14).

It is now known that *Igf2* affects the size of the placenta, and transfer of nutrients from mother to offspring, as well as foetal growth and resulting birth weight. Growth-promoting effects of this paternally expressed gene are directly countered by maternal expression of *Igf2r*, a fact established unequivocally in further mouse genetic studies (10). *Igf2r* is also known as cation-independent mannose-6-phosphate receptor and functions to sequester its ligands for lysosomal degradation. While mammalian *Igf2r*/mannose-6-phosphate receptor has a binding site specific for *Igf2*, the orthologous receptor in bird does not. All of these facts elegantly match the parent-offspring conflict hypothesis, in the mouse at least, and interaction between paternally derived *Igf2* and maternally derived *Igf2r* has been described as a 'parental tug-of-war' (15,16).

***Igf2* gene structure**

Igf2 gene in mammals, is comprised of a varying number of exons and promoters. It is transcribed and translated into a precursor hormone. After a number of processing steps, the end result is *Igf2*, in most species a 67 amino acid protein, although a number of variants arising from alternative splicing also occur. Different promoters and splicing patterns contribute to the complex regulation of *Igf2* expression and its effects in both foetal and adult tissues (Fig. 1). *Igf2* promoters and ex-

ons vary in number and length in mammals. Its various transcriptional isoforms emerge unevenly in different tissues and developmental stages and there are also considerable species differences. Promoter 0 (P0) (Fig. 1) is uniquely activated from the paternal allele in mouse placenta and also is crucial for normal growth in the womb (17). Human P0 is expressed in placenta, but also in foetal skeletal muscle and also in the adult, albeit at a lower level (18).

***Igf2* protein: function and receptors**

Translated *Igf2* protein is, in most species, a 67 amino acid protein that is related in sequence and structure to *Igf1*, insulin and relaxin [see (19) for review]. *Igf2* is a growth factor of particular importance for placental and embryonic growth. In mice, *Igf2* expression terminates in almost all tissues after birth. Mice have no adult-specific promoters, and knockout of *Igf2* P0-transcript leads to reduced placental size and subsequent foetal growth inhibition (17). An exception is the choroid plexus and leptomeninges of the brain, where *Igf2* is expressed biallelically and persists into adult life (8,20,21). In other mammalian species, including humans (22), pigs (23) and horses (24), *Igf2* protein is present in adult tissues as well. In addition, in sheep there is now evidence for a role for *Igf2* in foetal and placental development (25).

Igf type 1 receptor functions as a dimer similar to the two isoforms of the insulin receptor, *Insr-A* and *Insr-B*. These three receptors and the heterodimer *Insr-A/Igf2r* mediate proliferative and growth-inducing effects of both *Igf2* and *Igf1*, while binding of *Igf2* to *Igf2r* results in degradation. In mice *Igf2r*, is also imprinted, expressed only from the maternally inherited chromosome (Fig. 2), which has an important bearing on overall biological effects (16). However, whereas *Igf2* (and *IGF2*) is maternally imprinted (silenced) in both mouse (8) and humans (20), *IGF2R* appears to be non-imprinted in humans (26) and some other mammals.

Igf2 is produced locally in tissues in an autocrine or paracrine fashion and in liver, from where it is distributed *via* the blood in the endocrine manner. *In vitro* studies have shown that *Igf1* receptors are often expressed by the same cells that express *Igf2*, making an autocrine loop possible. *Igf2* expression studies in several species show transcriptional activity during development in several tissues, that support an autocrine or paracrine function (6). For example, human corneal cells exposed to recombinant *IGF2* enter S-phase preparing to divide, but do not express *IGF2* themselves. Cells in the posterior eye express *IGF2*, thereby illustrating paracrine mode of function (28). Moreover, studies in

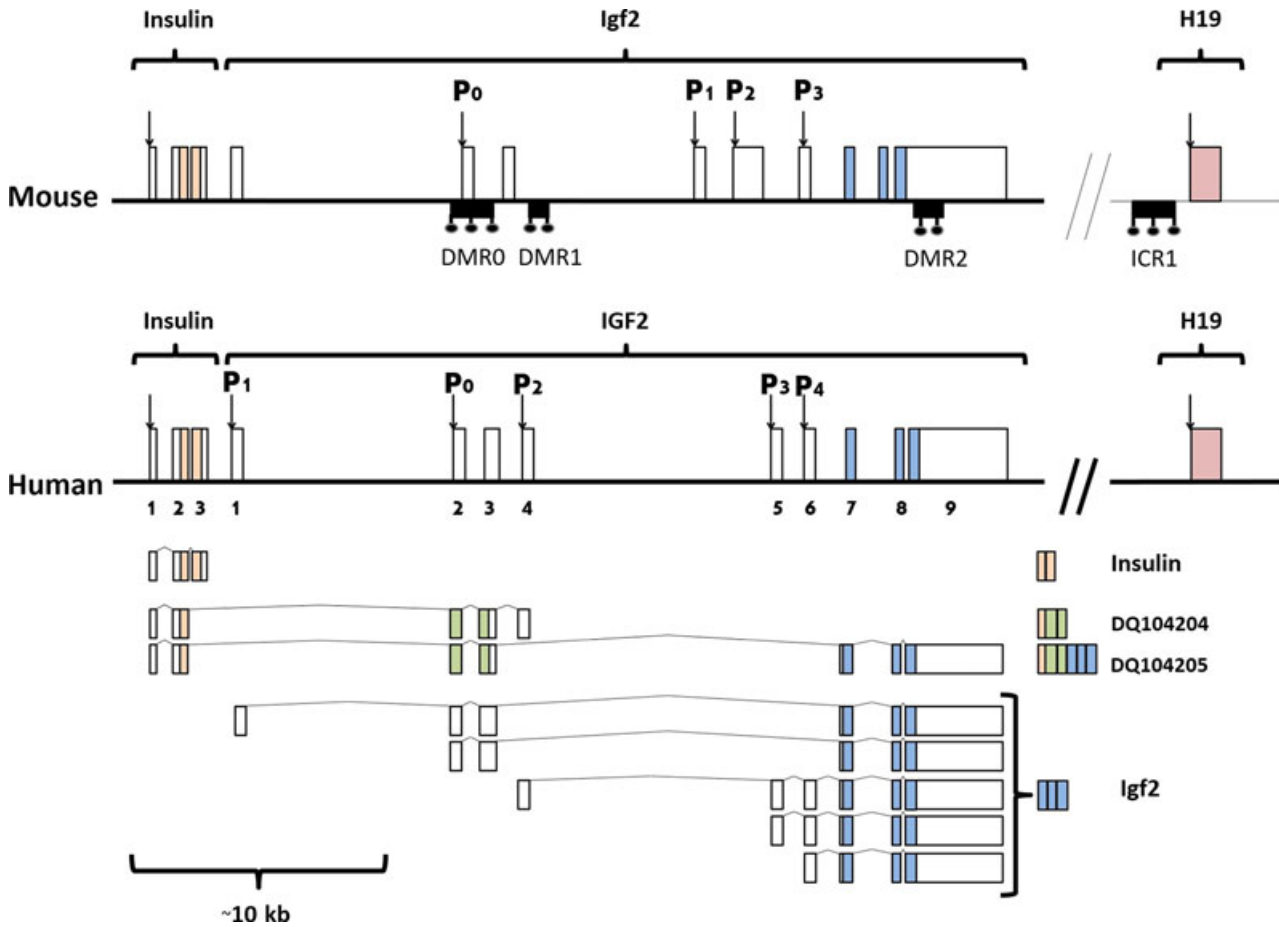


Figure 1. Mouse and human *Ins2/Igf2/H19* locus (top) and differently spliced human transcripts (bottom). Boxes above the line indicate exons; coloured exons are protein coding. Small, black boxes below the line represent differently methylated regions and the H19 imprinting control region. Pink checked H19 boxes represent a simplified scheme of a gene organized in several variable exons (so-called cassette exons) with intervening introns that differ between different species. Modified from Monk *et al.* (18).

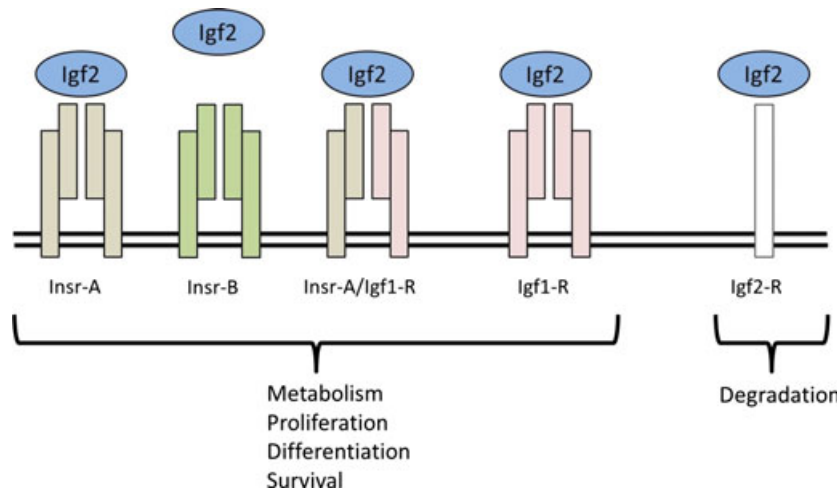


Figure 2. *Igf2* functions mainly through binding to *Igf1r* but can also bind, although with lower affinity, to both isoforms of the insulin receptor (*Insr-A* and *Insr-B*) and to the *Insr-A/Igf1r* heterodimer. Binding to these receptors induces various cellular responses, particularly proliferation and survival, mediated by *Igf1r*, while binding to *Igf2r* mediates internalization and lysosomal degradation. Adapted from Chao and D'Amore (27).

transgenic mice overexpressing *Igf2* suggest that growth effects are local to the sites of expression (29,30).

Igf2/H19 cluster and the role of *H19*

H19 gene is located immediately downstream of *Igf2* (Fig. 1). *H19* is transcribed, but not translated into protein. Both *Igf2* and *H19* are imprinted in a reciprocal manner in most somatic cells, where the paternal chromosome expresses *Igf2* but not *H19*, and the maternal chromosome transcribes *H19* but not *Igf2*. The *H19* gene encodes a 2.3 kb non-coding mRNA which is strongly expressed during embryogenesis. This gene belongs to an imprinted cluster, conserved on mouse chromosome 7 and human chromosome 11p15. *H19* and *Igf2* are oppositely imprinted and co-expressed in endo-derm- and mesoderm-derived tissues during embryonic development, which suggests a common mechanism of regulation. The role of *H19*, which lacks a conserved open reading frame, yet is abundantly expressed during mammalian development in both embryonic and extra-embryonic tissues, is intriguing. Lack of an open reading frame infers that *H19* functions as an RNA (31,32), however, apart from involvement of *H19* locus in imprinted regulation of *Igf2*, the physiological role of *H19* RNA has until recently been unclear. Evidence has been presented that *H19* can act as either an oncogene (33) or tumour suppressor (34–36) and that it can influence mouse growth independently of the mechanism co-regulating imprinted expression of *Igf2* and *H19* (37). It appears that *H19* RNA may influence growth regulation through at least two distinct mechanisms involving microRNAs (microRNAs consist of 19–25 non-coding nucleotides that have the ability to repress translation or promote RNA degradation). First, it has been shown that sequences within the first exon of *H19* are the source of microRNA miR-675, which is expressed exclusively in placenta and serves to suppress genes that promote

placental growth, including Igf type-1 receptor gene (*Igf1r*) (38). More recently, *H19* RNA has been shown to be a sink for let-7 family microRNAs (38). By sequestering let-7 microRNAs, *H19* was found to influence expression of let-7 target genes and promote differentiation of myoblasts (39).

Epigenetic modifications control gene expression

DNA methylation comprises addition of a methyl group (CH₃) to certain residues on the DNA, usually to a cytosine in a CpG dinucleotide. CpGs often occur in 'islands', with many CpGs congregated in a DNA sequence. DNA methyltransferases (Dnmts) are the enzymes that carry out DNA methylation. Some Dnmts use hemi-methylated DNA (newly replicated DNA where only one strand is methylated) as a substrate, adding a methyl group to C-residues. The old DNA methylation pattern is reproduced. Other Dnmts are responsible for *de novo* methylation, where unmethylated DNA is the substrate, and these Dnmts can create new DNA methylation patterns (Fig. 3). As a rule, genes with a methylated promoter cannot be expressed, albeit there are notable exceptions (40). The *H19* promoter is methylated on the paternal chromosome, inhibiting transcription of paternal *H19*.

Chromatin, by definition, comprises DNA associated with histone octamers that can be packed as dense heterochromatin or more loosely in euchromatin, depending on modifications that alter local structures and, as a consequence, influence gene expression. These modifications are exerted by enzymes that acetylate, deacetylate, methylate or demethylate specific histone amino-acid residues.

Igf2 and *H19* share enhancers

During development, *Igf2* and *H19* are concomitantly expressed in a variety of tissues (20). This has led to the

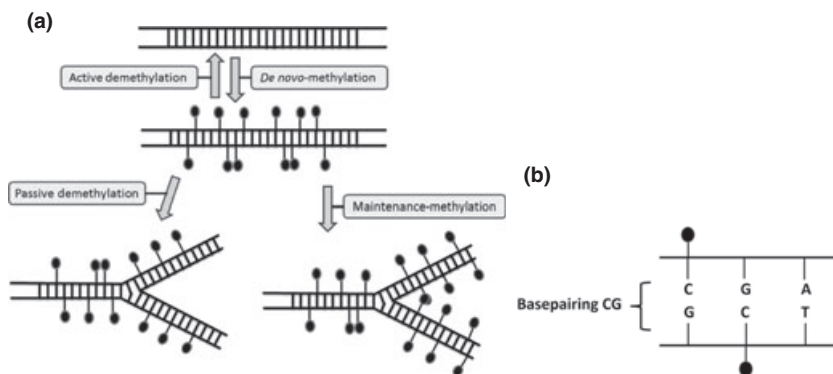


Figure 3. Some DNA methyltransferases (Dnmts) use hemi-methylated DNA (newly replicated DNA where only one strand is methylated) as a substrate, adding a methyl group to C-residues. Other Dnmts are responsible for *de novo*-methylation. Adapted from Reik and Walter (41).

idea of common enhancer elements that mechanistically link the genes together. Enhancers are short sequences of DNA that can bind transcription factors and enhance transcription level of genes. Leighton *et al.* (42) made a targeted deletion of two endoderm-specific enhancers, in mice, that lie 3' of *H19* as well as *H19* itself (43). Subsequent breeding showed that a maternally inherited enhancer deletion resulted in dramatic decline of *H19* in endoderm-derived tissues, including liver. Identical deletion on the paternally inherited allele resulted in an equivalent reduction of *Igf2* expression that was associated with growth impairment, so that mice were, at birth, about only 80% of normal weight, reflecting partial loss of *Igf2* expression. This elegant deletion experiment showed that these enhancers work on both alleles to promote either maternal *H19* expression or paternal *Igf2* expression.

Enhancer competition model

Differences in allelic methylation at *Igf2/H19* locus shown by Bartolomei *et al.* (44) revealed important information that was tested functionally in further transgenic mouse studies. The paternal *H19* allele was found to be methylated and inactive, while the maternal allele remained unmethylated from 900 bp upstream of the *H19* coding region to 700 bp downstream. Bartolomei *et al.* (44) ruled out the possibility that it was expression of *H19* that gave this parental-specific pattern by comparing different tissues, including foetal liver, where *H19* is maternally expressed, adult liver where maternal *H19* expression has ceased, and adult brain where *H19* is not expressed, as well as embryonic stem cells, that do not express *H19*. In all tissues, the maternal allele was unmethylated and had open chromatin structure in the region of the *H19* promoter, independent of expression pattern. Two downstream enhancers were open in chromatin structure on both parental alleles. Bartolomei *et al.* (44) combined these findings with data from Sasaki *et al.* (45) concerning allele-specific methylation in a *DMR* sequence 5' to *Igf2* and presented an 'enhancer competition model' that could explain reciprocal imprint of both *Igf2* and *H19*. The hypothesis was that enhancers could work on either *Igf2* or *H19* and suggested that *H19* methylation on the paternal allele was the key determinant of *H19* silencing and *Igf2* activation.

Two transgenic mouse lines containing the *H19* domain including 4 kb 5' and 8 kb 3' DNA were examined regarding parental-specific methylation and *H19* expression. Homozygous male and female mice were bred to non-transgenic mice. Liver cells from progeny typically exhibited methylation and expression patterns

similar to endogenous *H19*, were hypermethylated and silent when inherited from the father and unmethylated and expressed when maternally derived. A few maternally inherited transgenes exhibited a hypermethylated and unexpressed pattern, that typically found in paternally inherited *H19* transgenes, possibly due to insufficient sequence upstreams of *H19*, or perhaps due to influence of endogenous sequences at the transgene insertion site, or some other unknown influence on the imprinting process. Even so, these results showed that information contained within the *H19* domain transgene, including a region of 2 kb located 4–6 kb upstream of *H19* transcription, was enough to confer parental-specific methylation and expression patterns after passage through the germ cells. The result indicates that it is not the methylation itself that is the parental mark, but that the passage through the germline cells somehow establishes these differences. It also showed that enough information for parental-specific expression of *H19* is localized in the vicinity of the gene itself.

The enhancer competition model attempted to explain reciprocal imprinting of both *H19* and *Igf2* (Fig. 4). It predicted that enhancers could work on either *Igf2* or *H19* and suggested that methylation of the paternal *H19* inhibits expression and thus gives enhancers a chance to work on the *Igf2* gene. However, it could not explain subsequent findings concerning differently methylated domain 5' to *H19*, called imprint control region 1 (*ICR1*).

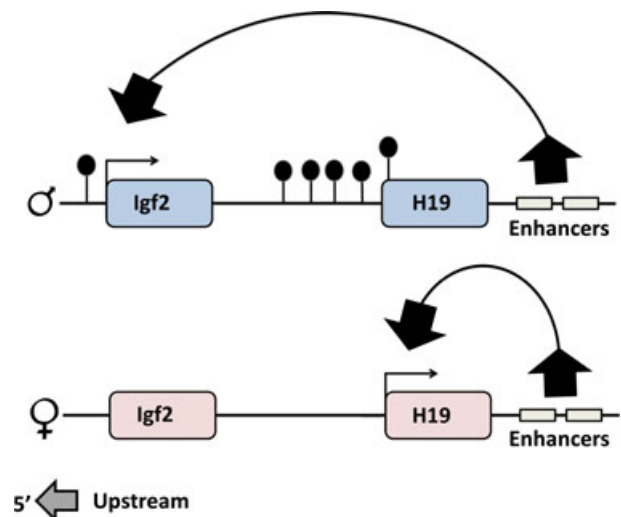


Figure 4. The enhancer competition model to explain imprinted regulation of *Igf2* and *H19*. Big boxes represent genes on the paternal (top) and maternal chromosome, with arrows indicating gene expression. CH3-lollipops represent regions containing methylated cytosine residues. Small boxes show position of downstream enhancers.

Igf2/H19 imprint control region

Human chromosomal region 11p15 contains two independent imprinted regions controlled by two *ICRs*, *ICR1* and *ICR2*. One region, located approximately 2–4 kb upstream of *H19* transcription start site has been shown to be important to the imprinting state of both *H19* and *Igf2* and is referred to as *ICR1*. *ICR1* corresponds to a region of differential methylation, also known as the *H19* DMD since it is rich in CpG residues that differ in state of methylation on the two alleles, with the paternal allele being methylated (Fig. 5). The unmethylated maternal allele enables binding of an insulator protein CTCF, which prevents common enhancers acting on *Igf2*. *ICR1* is essential for imprinting of *H19* *in vivo*, since mice with deleted *H19 ICR1* lose their imprint (46). Paternal *H19* alleles were expressed to a level of approximately half that of wild-type maternal *H19* expression. Parental-specific DNA methylation was lost in a short, remaining sequence of *ICR1* and in the sequence between the *DMD* and the *H19* promoter. This and subsequent studies showed that *H19 ICR1* is required for *H19* transcription and to prevent downstream enhancers from accessing *Igf2* promoters on the maternal chromosome, and that *ICR1*-dependent transcription of *H19* is required for maintenance of key epigenetic marks (46,47).

Light has also been shed on this issue by studying two rare disorders caused by alterations in the 11p15 region in humans, Beckwith–Wiedemann Syndrome (BWS) (48,49) and Silver–Russell Syndrome (SRS) (50). BWS was one of the first syndromes in which overexpression of IGF2 was coupled to a growth disorder. BWS also provided the first link between overgrowth and tumour development (48,49). BWS can occur as a result of alterations in *ICR1* as well as *ICR2*. First, simple methylation of maternal *ICR1* has been found to account for 5% of BWS cases. Secondly, *ICR2* is normally paternally imprinted and thus regulates

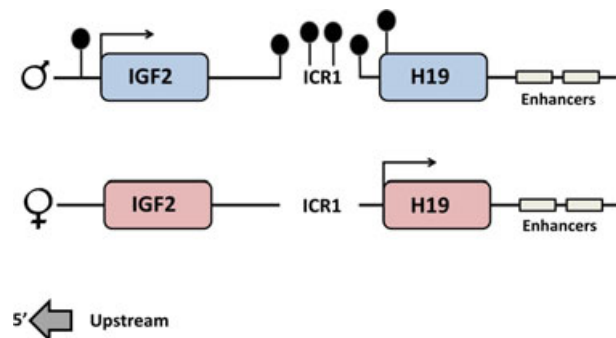


Figure 5. Parental-specific DNA methylation of *ICR1* is required for the imprinted status of both *H19* and *Igf2*. Adapted from Chao and D'Amore (27).

expression of maternally expressed *CDKN1C*, a growth regulatory gene. *ICR2* resides adjacent to a non-imprinted gene, *KCNQ1* and is the promoter for *KCNQ1OT1*, a paternally expressed RNA whose expression regulates *CDKN1C* in cis (51). Hypomethylation of *ICR2* is the most frequent genetic aberration in BWS. In parallel, it has been shown that SRS patients display aberrations including hypomethylation of *ICR1* as well as *ICR2* (50). Opposing epigenetic lesions in BWS and SRS are consistent with their defining features as disorders of overgrowth and growth restriction, respectively.

CTCFs and the boundary model

The *ICR1* sequence contains direct and indirect repeats, including several CAGCCC motifs, discovered in human *H19 ICR* as early as 1987 (52). CTCFs are specific zinc-finger proteins that bind to CAGCCC and related sequences to influence gene expression at numerous loci, both imprinted and non-imprinted. Wang *et al.* (53), using ChIP-seq, identified 77811 separate CTCF-binding sites in the human genome. The 19 cell types examined exhibited marked variability, with average number of bound CTCF sites being in the order of 55 000. DNA methylation is one factor that can prevent CTCF binding, with 41% variation of CTCF binding found to be due to differences in DNA methylation.

Dixon *et al.* (54) used the Hi-C method to reveal information concerning three-dimensional architecture of chromosomes. CTCFs were concentrated on boundaries of topological domains, along with transfer RNAs, short interspersed elements and housekeeping genes, indicating that these are necessary for establishment of the three-dimensional architecture of genome domains in cell nuclei. An updated genome-wide CTCF-binding site database includes calculations of distance from CTCF sites to nearest domain boundary, thus allowing further research on how CTCF-binding sites act to organize the three-dimensional structure of DNA (55). Following the demonstration that CTCF-mediated chromatin organization at the *Igf2/H19* locus is dependent on cohesin (56), it has become clear that CTCF and cohesin-mediated chromatin interactions are important for gene regulation at many loci (57).

Bell and Felsenfeld (58) and Hark *et al.* (59) suggested that CTCF binding on maternal *ICR1* blocks downstream enhancer sequences from accessing *Igf2* promoters and thereby silences maternal *Igf2* expression, while paternal-specific *ICR1* methylation prevents CTCF binding and is permissive for paternal *Igf2* expression (Fig. 6). This enhancer-blocking, or boundary function of *ICR1* was confirmed by different approaches, making clear that *ICR1*-bound CTCF functions as a genomic

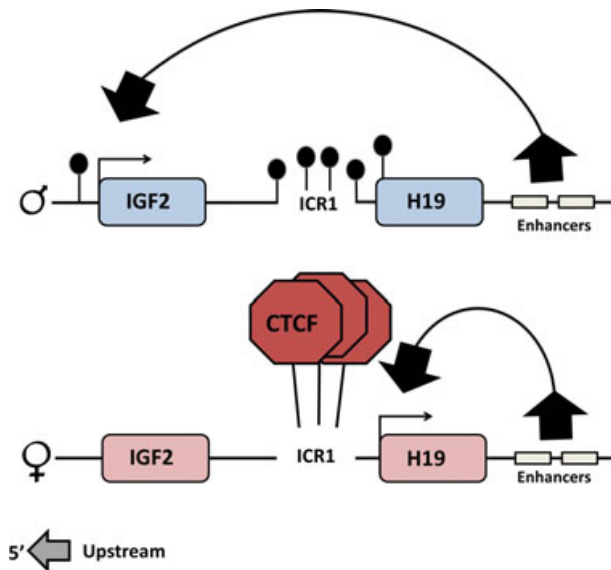


Figure 6. The boundary model of imprinted regulation of *Igf2* and *H19* states that binding of CTCF proteins on maternal *ICR1* works as an insulator preventing enhancer elements from acting on the *Igf2* maternal allele. Adapted from Chao and D'Amore (27).

insulator and that *ICR1* position between *H19* and *Igf2* made this possible. The enhancer competition model was ruled out, since it could not explain why *ICR1* position between the two genes was essential. Conservation of the cluster was to some part explained since the cluster could not be regulated if genes, insulator binding domains or enhancers were not in the correct order. Hark *et al.* (59) were more specific when analysing CTCF binding on methylated DNA; they analysed hemi-methylated DNA and found that only hemi-methylation on the top strand of DNA inhibits CTCF binding, while bottom-strand methylation was unimportant. In replicating cells, this means that the paternal allele transiently has one hemi-methylated bottom strand where CTCF proteins could in theory bind, but (at least in normal cells) does not. Involvement of CTCF in the boundary model added components to the imprinting puzzle, but it was still not clear whether methylation at *ICR1* was fundamental for establishing imprinted expression.

Histone acetylation and DNA methylation affect gene expression

Pedone *et al.* (60) immunoprecipitated cells with antibodies against acetylated histone 3 (H3) and H4 tails to detect hypoacetylated histone tails, commonly found in dense chromatin and hyperacetylated tails, associated with chromatin open for transcription (Fig. 7). The silent

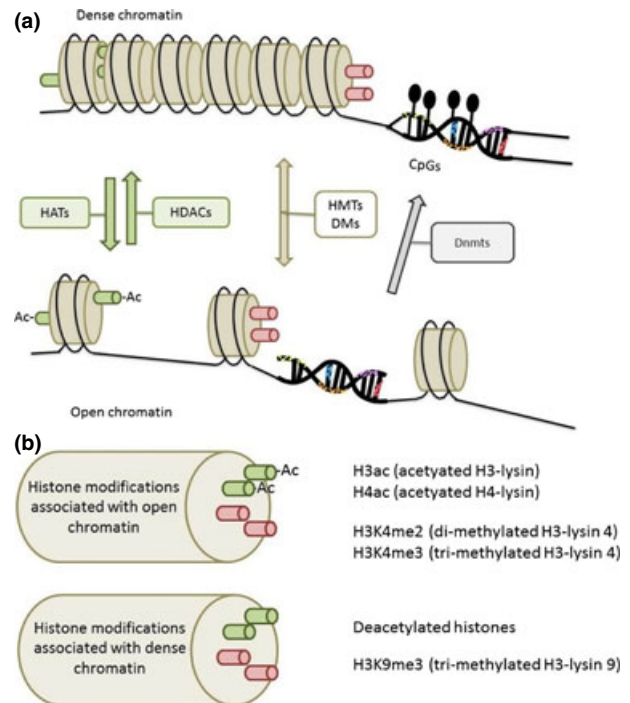


Figure 7. Chromatin regulation mediated by histone modifications. (a) Histone acetyltransferases (HATs), histone deacetyltransferases (HDACs), histone methyltransferases (HMTs), demethylases (DMs) are enzymes involved in forming open or dense chromatin. (b) Some key histone modifications associated with open or dense chromatin states. Model created with data from Verona *et al.* (61).

paternal *H19* allele was hypoacetylated compared to the maternal allele, but *Igf2* alleles were equally acetylated. These authors (60) also cultured cells either in the presence of DNA methylation inhibitors (leading to reduced DNA methylation), or inhibitors of histone deacetylases (leading to more open chromatin), or in a medium with both, and these treatments were found to affect the imprints. *H19* imprint was lost only when both inhibitors were added, whilst *Igf2* was biallelically expressed when either or both inhibitors were present. These findings suggest that both DNA methylation and histone modification are important for maintenance of the *Igf2* imprint.

Are chromatin differences a consequence of transcriptional status?

Verona *et al.* (61) characterized histone modification in imprinted regions, including the *H19 ICR1* region on both parental alleles. Allele-specific histone acetylation and histone methylation were observed and active maternal *H19* allele was associated with active chromatin modifications (described in detail in Fig. 7b). The paternal *H19* allele was in a dense chromatin state. *Igf2* was

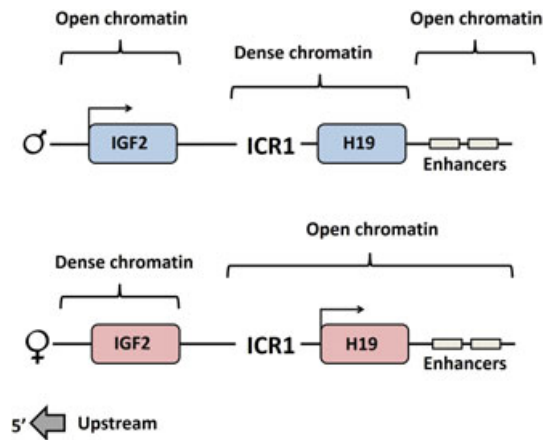


Figure 8. Summary of chromatin differences at the *Igf2/H19* paternal and maternal alleles, according to Verona *et al.* (61).

reciprocally in a state of low chromatin density while enhancers were open (Fig. 8). Highest level of 'active histones' was found at the maternal *H19* promoter. Differences in *ICR1* raised a question: do specific chromatin modifications in *ICR1* allow transcription or are they a consequence of transcription? To answer this, Verona *et al.* (61) compared an *H19* gene with deleted *ICR1* in tissues where *H19* was not expressed, to neonatal liver where it was expressed (even without the *ICR*). The result was 'active' chromatin at *H19* in neonatal liver, but not in other tissues, suggesting that it is transcription level that provides the allele-specific chromatin pattern. Thus, differences in chromatin are not an effect of some imprinted mark but an effect of transcriptional level. These authors did not rule out that observed allele-specific DNA methylation and chromatin differences could be interconnected and reinforcing each other.

Parent-specific loops and the chromatin loop-model in mice

Murrell *et al.* (62) made a targeted insertion of *Igf2/H19*-genes to generate a mouse where the genes and promoters showed activity, ascribable to a normal cell. They used a chromosome conformation capture technique that enabled analysis of physical organization of specific chromosomal regions in the nucleus. Murrell *et al.* (62) reported parental allele-specific differences that involved differentially methylated region 1 (DMR1) and DMR2 within the *Igf2* gene (Fig. 9), showing that maternal *ICR1* interacts with *Igf2* DMR1, and paternal *ICR1* meshes with *Igf2* DMR2. This generated a three-dimensional model that provided a simple epigenetic explanation for imprinted expression of *Igf2* and *H19*: DMRs and ICRs contain insulators, silencers and activa-

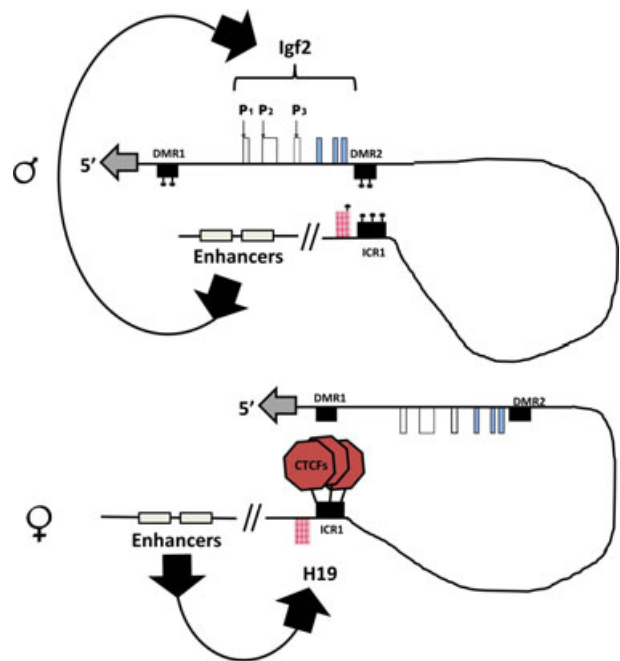


Figure 9. Chromatin looping at paternal and maternal *Igf2/H19* alleles. DMR1, DMR2 and ICR1 are methylated on the paternal chromosome, and putative proteins bind methylated ICR1 to DMR2, bringing downstream enhancers into close proximity with the paternal *Igf2* promoters. On the maternal chromosome, ICR1 binds CTCFs (also cohesion and likely other proteins) and this promotes interaction with DMR1, creating an inactive domain where maternal *Igf2* has no access to enhancers. Pink checked *H19* boxes represent a simplified scheme of a gene organized in several variable exons (so-called cassette exons) with intervening introns that differ between species. Modified from Murrell *et al.* (62).

tors and are turned on with differential methylation that enables or inhibits expression of the genes.

Germ cells carry parental information

Methylation prevents CTCF binding on the paternal *Igf2* allele (63,64), but how is methylation established? According to earlier findings (44), correct parental methylation pattern is an effect of passage through either the female or the male germline cells in an embryo, and thus probably not to the imprinted mark itself.

In a developing embryo, some cells become primordial germ cells (PGCs), still diploid but destined to migrate to the gonads, proliferate and differentiate into haploid sperm or oocytes. Epigenetic status of these cells changes dramatically before meiosis. PGCs undergo demethylation, where most DNA methylation is erased, including imprinted areas. Parental differences in modification of histones and chromatin are also removed before meiosis. So far, there is no definitive explanation

for the observed demethylation and removal of chromatin differences. It is not known if demethylation causes removal of chromatin modifications, or alternatively if chromatin modifications are a necessary prerequisite for demethylation (65–68). Demethylation of haploid PGC is a necessary step, since all old parental methylation needs to be erased, so that all alleles can acquire new methylation patterns appropriate to the sex of the embryo in which they are developing. Alignment and recombination of homologous chromosomes during meiosis is not possible without the demethylation (63).

In mouse development, between 10.5 and 12.5 dpc, patterns of DNA methylation in imprinted and testis-specific genes are erased in PGCs (63,64). In the male, DNA methylation begins to be reestablished around 15.5 dpc for imprinted genes (65–67).

Concluding remarks

Imprinted expression of Igf2 has evolved in mammals, but why?

The silent maternal *Igf2* copy has no effect on foetal growth; however, in mice, maternal *Igf2r* acts as a direct antagonist of *Igf2* by targeting it for lysosomal destruction. It is in the mother's interest to provide her offspring with sufficient resources for their growth and subsequent survival, with resources allocated evenly to the offspring throughout her reproductive span. In species where one female will typically reproduce with more than one male, it is in the interest of the male to promote resource allocation to his offspring in a more opportunistic manner. This difference in parental interests was recognized by Haig and Westoby (4) and is known as the 'parent-offspring conflict theory' to explain evolution of imprinted genes. It is accepted that for imprinted gene expression to evolve there must be benefits that outweigh cost, or risks, associated with silencing one of the two parental alleles of a gene, notably that a single mutation in the active copy may abolish all the gene's function. The conflict theory appears to hold true for at least a subset of imprinted genes that function to regulate foetal growth and can explain why genomic imprinting is essentially confined to mammals and flowering plants, in which the placenta and endosperm serve similar functions in nutrient provision from mother to offspring during foetal growth. The theory has been extended, for instance, to consider behaviour of imprinted genes in populations but does not obviously accommodate all the 100 or so genes that are now known to be imprinted and have diverse functions, including in adult physiology and behaviour (11,13,69).

In whose interest is this paternal-specific expression?

It is unclear how imprinted parental-specific gene expression arose, and the question remains who benefits from it. Paternal-specific *Igf2* expression is explainable only if there are differences in *Igf2*-allele efficacy. Assuming some *Igf2* alleles are more demanding of maternal resources and others less, then selective pressure supports the more resource-demanding paternal-specific expression of *Igf2*. Likewise, it is assumed that, at least in mice, selective pressure also supports *Igf2r* alleles that are more effective at neutralizing *Igf2* and expression from the maternal *Igf2r* allele. Ultimately, these antagonistic control overgrowth must be in balance to achieve an optimum for offspring survival and fitness. The balance must work at population level, as well as at the level of individuals, and this is recognized by extension of the parent-offspring conflict hypothesis to include the idea of kinship, or relatedness within populations (69).

It is commonly assumed that there exists interbrood competition for resources *in utero*. In theory, this could be countered by females reallocating resources within a litter or conserving resources for investment in future broods. Mouse manipulation experiments have shown that females that had invested lightly into the first brood did not invest more heavily into the second brood than females that invested more heavily into the first brood (70). It was therefore concluded that females are somehow 'primed' by their experience in their first brood so that number in the second brood is determined in part by number in the first (70). In addition, there may also exist a trade-off between litter size and size of individual offspring, so that reproductive success may benefit under some circumstances from larger litters with smaller offspring, and in other instances from smaller litters of larger offspring. There is evidence of this from manipulation in mice of another imprinted gene, *Grb10*. *Grb10* encodes a signalling adapter protein from the maternal allele, expressed widely throughout foetal development, that normally acts to limit placental as well as embryonic growth. Although *Grb10* knockout mice are at birth significantly larger than their wild-type littermates, mothers carrying disrupted maternal *Grb10* allele had larger litters and smaller offspring than those inheriting disrupted *Grb10* allele from the father. It was therefore concluded that there is a grandparental effect whereby *Grb10* can affect reproductive strategy by allocating maternal resources, so as to influence the relationship between offspring number and size (71).

The operational role of *Igf2* protein is only partly known. Even though we have gathered considerable information concerning structure and function of the

gene, the time is ripe to link this information to how the peptide participates in both normal growth development and pathogenesis of key diseases (19,72,73). There are clearly one or more epigenetic marks that distinguish parental alleles, and there are key element(s) that make parental-specific expression possible. Imprinting of the *H19/Igf2* gene cluster is important in many different cancers and syndromes including growth disorders, and deeper knowledge of imprinting mechanisms could enable development of diagnostic tests and possible future therapies.

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