Imprinting and the Epigenetic Asymmetry Between Parental Genomes

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Genomic imprinting confers a developmental asymmetry on the parental genomes, through epigenetic modifications in the germ line and embryo. These heritable modifications regulate the monoallelic activity of parental alleles resulting in their functional differences during development. Specific *cis*-acting regulatory elements associated with imprinted genes carry modifications involving chromatin structural changes and DNA methylation. Some of these modifications are initiated in the germ line. Comparative genomic analysis at imprinted domains is emerging as a powerful tool for the identification of conserved elements amenable to more detailed functional analysis, and for providing insight into the emergence of imprinting during the evolution of mammalian species. Genomic imprinting therefore provides a model system for the analysis of the epigenetic control of genome function.

Genomic imprinting, unique to mammals among the vertebrates, is a phenomenon that causes some genes to be expressed according to their parental origin and results in a developmental asymmetry in the function of parental genomes. Imprinted genes have roles in prenatal growth, development of particular lineages, and in behavior, as well as being implicated in human diseases (*1, 2*). Imprints are initiated during gametogenesis and are inherited by mature gametes and then transmitted to embryos (*1–4*).

DNA methylation by the enzyme Dnmt1 plays a central role in genomic imprinting. Appropriate imprinting is lost for many genes in *Dnmt1* null embryos, although indirect effects cannot be discounted, and at least one imprinted gene, *Mash2*, remains unaffected (*5, 6*). Genomic imprinting also occurs in marsupials, and current knowledge suggests that DNA methylation may not be involved (*7, 8*) however, further work is necessary to confirm this. Traditionally, chromatin modifications and DNA methylation have been considered as largely separate entities, but recent advances in identifying epigenetic mechanisms informs us of potential connections between imprinting, chromatin structure and DNA methylation. Links between general epigenetic inheritance mechanisms and chromatin structure and function also include those between histone methylation and the heterochromatin protein HP1 that operate widely, for example, in yeast and mice (*9–13*). What is entirely unknown, is how the methylation of imprinted genes is initiated during gametogenesis starting from the "ground state." Chromatin structural modifications in conjunction with DNA methylation seem a plausible mechanism.

The Genesis of Imprinting

Speculation about the emergence of genomic imprinting during evolution has arisen from the study of "foreign" sequences such as transgenes and parasitic repetitive elements, including retrotransposons, retroviruses, and some repetitive elements, which are shut down by epigenetic modification. The majority of 5-methylcytosine lies within these elements (*14, 15*). This methylation facilitates a host defense mechanism to counter the potentially deleterious effects of such elements: Promoter methylation of the elements inhibits their transcription and methylation directed heterochromatinisation of repetitive elements is proposed to prevent chromosomal rearrangement. Interestingly, the two parental germ lines might methylate such parasitic elements differentially in a class-dependent manner (*16, 17*). While these elements have methylation patterns characteristic of imprinting regulatory regions, some usually become methylated on both alleles soon after implantation.

However, some classes of repetitive elements that exist even transiently as differentially methylated regions (DMRs) may impose epigenetic influence on neighboring sequences and genes. Consistent with this is the finding of differentially methylated tandem repeat sequences adjacent to many imprinted genes or imprinting regulators (*18*). In another type of example, 16% of transgenic sequences randomly incorporated into the mammalian genome become imprinted (*19*). Interestingly, a retrotransposon-derived gene, *Peg10*, is itself imprinted (*20*). In addition, an allele at the *agouti* locus in the mouse exhibits parental origin effects imposed by a neighboring *IAP* retroviral element (*21*). Taken together, these observations contribute to the hypothesis that the initial germ line methylation of "parasitic" repetitive sequences could act as primary parental imprinting signals. This putative relationship contributes to the theory that genomic imprinting evolved through an adaptation of the host defense system when some selective advantage was attained by an imprint mark from a repetitive element extending to a nearby gene (*22*). This theory awaits experimental validation.

The emergence of genomic sequence data from different mammalian species is assisting in the search for patterns, location, and classes of such elements in imprinted domains. Indeed, some imprinted domains contain conserved features including tandem repeats and retroviral-like sequences (*18, 23, 24*), whose role can now be investigated. Such comparative approaches could be extended to marsupials and oviparous monotremes (Platypus and Echidna), because imprinting is evident in the former but not the latter (*7, 25*). In this way, evolutionary links involved in imprinting can be sought in the appropriate extant regions. Interspecies genomic sequence comparisons at known imprinted loci will continue to facilitate the characterization of these regions. Such comparative sequence analysis has already been used to identify new genes within imprinted clusters, as well as conserved putative regulatory elements (*26–28*). These studies demonstrate the power of bioinformatics in imprinting research.

Epigenetic Control of Imprinting Regulatory Elements

A number of stereotypical arrangements of imprinted genes are beginning to emerge (Fig. 1). In general, allele-specific DMRs are a hallmark of imprinted genes and they can be associated with different functions (*29*). For example, about half of the identified imprinted genes are clustered with long-range *cis*-acting imprinting centers (IC) (*1, 2, 29*). These ICs carry allele-specific methylation marks, which are established in the germ line and retained thereafter (*2, 29*). However, their modes of

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action may differ. ICs subsequently influence epigenetic modification of additional *cis*-acting regulators important in allele-specific, tissue-specific, or temporal-specific regulation of imprinted genes (*30*). Closely linked maternally expressed and paternally expressed imprinted genes can coexist, although differential methylation within a cluster is usually biased toward only one of the parental chromosomes (*2, 29, 30*).

A particularly interesting recent finding is the apparent parallel between the *Igf2-H19* and the *Dlk1-Gtl2* loci, which share many common features (*31, 32*) (Table 1). These are closely linked reciprocally imprinted pairs of genes, the former being one of the best-characterized imprinted regions to date. It is clear from gene and sequence analysis that the two pairs do not represent locus duplication, and while aspects of imprinting control may be the same it is expected that other elements may be specific to each pair (*33*). This relationship allows a comparative analysis of the two domains and has the potential to contribute further to an understanding of imprinting regulation through the identification of shared and unique elements involved in both short and long-range imprinting control.

Silencing of the *Igf2* allele in the absence of methylation is explained by insulation and silencing mechanisms (Fig. 1) (*2, 30*). Allele-specific methylation at the *H19* promoter contributes to the silencing of its paternal allele, however the *Igf2* promoters of the silent maternal allele are unmethylated (Fig. 1). The reciprocal imprinting activity of *Igf2* and *H19* is regulated by a differentially methylated IC, located 2 to 4 kb upstream of *H19* (Fig. 1). This IC can also function as a methylation-sensitive insulator that binds the factor CTCF on the unmethylated maternal allele. This prevents downstream enhancers from interacting with the upstream maternal *Igf2* promoters. CTCF binding is abolished by DNA methylation of the IC on the paternal chromosome, thus allowing the downstream enhancers to interact with the paternal *Igf2* promoters (*34–37*). Enhancers for choroid plexus and leptomeninges where *Igf2* is not imprinted, hence biallelically expressed, are located upstream of the insulator, so they are not affected by CTCF binding (*38*) (Fig. 1). The insulator model has been shown to function primarily in endoderm. However in mesoderm, an alternative or an additional mechanism involving a silencer element either modulates insulator function or acts independently of the insulator to regulate maternal *Igf2* silencing (*30, 39*). For example, the *Igf2*-DMR1 (Fig. 1) acts as a tissue-specific silencer, most likely through binding a repressor protein on the less methylated maternal allele (*30, 39, 40*). These results invoke a new role for epigenetic regulation of gene expression involving DNA methylation.

Another type of arrangement regulating imprinting involves antisense transcripts (Fig. 1). For example, *Igf2r* contains two DMRs, one is a maternal germline-inherited intronic methylation mark associated with an antisense transcript expressed only from the paternal unmethylated intron. Antisense transcription extends through the promoter of the *bonafide* transcript on the paternal allele and may mediate silencing (*41, 42*). On the maternal allele, the antisense transcript is repressed by intronic methylation, which permits transcription from the maternal *Igf2r* promoter. Later in development, the second DMR at the promoter of the paternal allele, becomes methylated perhaps as a consequence of allelic inactivity. In humans, *IGF2R* expression is not usually imprinted, consistent with a lack of sequence conservation with the mouse intronic DMR and an apparent lack of an antisense transcript (*43, 44*). This adds further support for a role for the antisense transcript in *Igf2r* imprinting in mice. Recent evidence suggests that the maternally expressed *Ube3a* gene, located at the end of a large cluster of paternally expressed imprinted genes (Fig. 1), may also be regulated by an antisense transcript expressed from the paternal chromosome (*45*). Data suggests that other imprinted genes may be similarly regulated (*46*).

A Reductionist Approach to Imprinting Regulation

If the *Igf2-H19* domain is a good paradigm, the chromosomal domains containing imprinted genes are complex entities with multiple and hierarchical regulatory elements situated

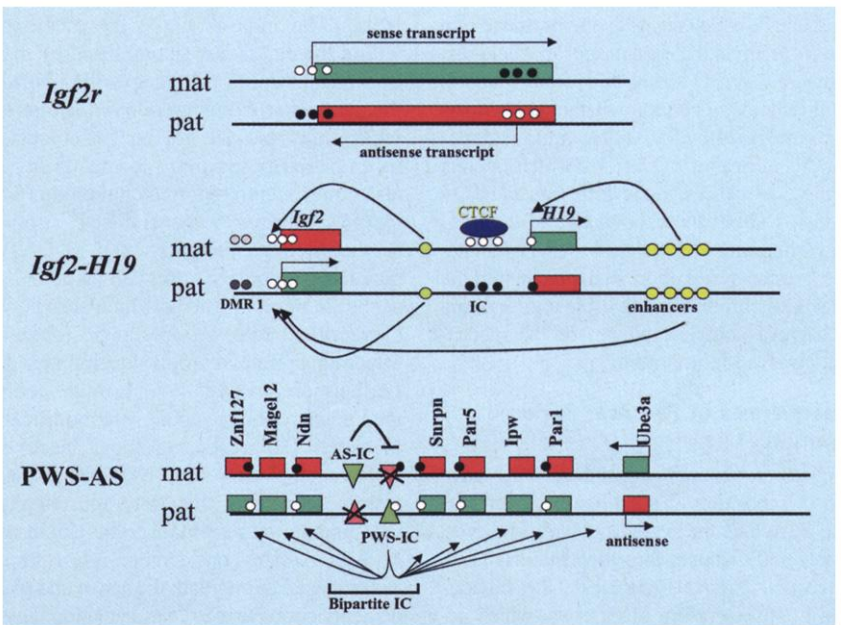

Fig. 1. Schematic representation of epigenetic regulation at three different imprinted loci. The *Igf2r* locus contains a single imprinted gene regulated by an antisense transcript, which itself is regulated by a differentially methylated germline imprint located in an intron. The *Igf2-H19* locus contains a pair of reciprocally imprinted and coordinately regulated imprinted genes controlled by an intergenic imprinting center (IC), which binds CTCF when unmethylated and insulates the *Igf2* fetal promoters from common downstream enhancers in endoderm. In mesoderm, tissue-specific silencers such as DMR1 play a role in *Igf2* regulation—mesoderm enhancers have yet to be identified. Tissue-specific enhancers upstream of the insulator regulate biallelic activity of *Igf2* in parts of the brain. A larger gene cluster containing multiple imprinted genes is regulated by a bipartite imprinting center associated with the Prader-Willi/Angelman Syndrome locus (PWS-AS) on human chromosome 15q/central mouse chromosome 7. Here, a bipartite *cis*-acting imprinting center confers long-range imprinting control on the two parental alleles. Female germline transmission of an AS-IC is required for methylation and repression of the maternal alleles of the paternally active imprinted genes through inactivation of the PWS-IC. On the paternal chromosome, this AS-IC is nonfunctional allowing the PWS-IC to confer paternal allelic expression on upstream and downstream genes. *Ube3a* is expressed from the maternal allele in the brain and appears to be associated with an antisense transcript on the paternal allele in a manner similar to the imprinting of *Igf2r*. Recent data suggests that the PWS-IC is required for expression of the antisense transcript; and in its absence, the antisense is repressed and the paternal allele expresses the *Ube3a*. White circles denote absence of methylation at a differentially methylated region and black circles, methylated regions. Gray circles indicate partial methylation. Larger yellow circles denote enhancers. Active alleles of genes are noted in green with silent alleles in red. Arrows indicate interactions between *cis*-elements on the two parental chromosomes. Drawings are not to scale (*2, 29, 30, 45*).

among widely dispersed enhancers. Until recently, it has proved difficult to obtain reliable imprinting of small transgenes, such as *H19,* a prerequisite to more detailed investigations (*47*). Remarkably, a 1.2-kb mini-transgene derived from the paternally expressed *Snrpn* gene located in the Prader-Willi (PWS)/Angelman (AS) Syndrome imprinted gene complex (Fig. 1) was shown to undergo imprinting. The transgene which contained a 200-bp *Snrpn* promoter/exon1 fragment (putative paternal PWS-IC) and a 1-kb sequence located 35 kb upstream of the *SNRPN* promoter (putative maternal AS-IC) (*48*) resulted in differential methylation, appropriate monoallelic paternal expression and characteristic asynchronous replication (Fig. 1). A PWS-IC transgene lacking the 1-kb sequence became methylated irrespective of parental origin. Hence, the AS sequence could therefore be seen to "protect" the transgene from being methylated upon paternal inheritance. The intronic DMR of *Igf2r* has also been analyzed in further detail, which suggests the presence of a de novo methylation signal and an allele-discrimination signal within the critical region (*49*). It remains to be seen whether such bipartite examples extend to other imprinted domains. Identification of factors, which interact with these sequences, in the germ line and early embryos, will advance knowledge of the initiation of imprints and their subsequent maintenance. Further verification, in the context of the endogenous chromatin is feasible and essential to achieve a complete picture of the mechanisms involved in imprinting.

Consequences of Parental Genome Asymmetry

Evolution of genomic imprinting must have required imposition of additional requirements on the germ line, the zygote and early embryos, to create and maintain the epigenetic asymmetry between parental genomes. One obvious necessity is the erasure of imprints, which occurs in primordial germ cells (*50–52*) before reinitiation of new imprints during gametogenesis. Primordial germ cells have a dominant activity capable of extensive demethylation and erasure of imprints in a somatic nucleus (*53*), but the precise mechanism is unknown.

The oocyte cytoplasm is also deployed to discriminate between parental genomes during evolution, as seen in the selective elimination of paternal chromosomes in some lower organisms, and in the exploitation by "selfish" endosymbiotic bacteria to manipulate host reproduction through generating interspecific barriers, causing parthenogenesis or even feminization of genetic males (*54*). In this context, it is striking that in mammalian fertilized eggs the paternal genome undergoes dramatic demethylation, while the maternal genome is not affected in this way (*55*). This phenomenon is apparently absent in lower vertebrates and may have evolved according to the "genomic conflict" hypothesis (*56*). These early events may also facilitate early activation of the embryonic genome typically seen in mammals, or be associated with early events important for later placentation. The importance of the environment within the egg is also exemplified by disruption of imprinting in interspecific hybrids of the deermouse *Peromyscus maniculatus* (*57*), which may be due to nuclear-cytoplasmic incompatibility. Similar incompatibility may also contribute to reported cloning inefficiency (*58*). Oocytes are generally very complex as they contain proteins, such as OCT4 to guarantee totipotency, as well as others such as the *Polycomb* proteins which are known to play critical roles in epigenetic inheritance affecting preimplantation development (*59*). Furthermore, oocyte cytoplasmic modifiers are known to induce epigenetic modifications of target loci rendering them inactive by DNA methylation (*60, 61*). Their mechanisms of action at this early and crucial developmental stage remain to be elucidated.

An important consideration is how imprints are protected during both the active and passive demethylation during preimplantation development since, at least some of the germ line methylation imprints are resistant to genome-wide demethylation events (*2, 3*). In this context, recent evidence shows that the preimplantation embryo contains a remarkable oocyte-specific form of Dnmt1 which enters the nucleus transiently at the eight-cell stage and ensures maintenance of DNA methylation associated with parental imprints (*62, 63*).

A consequence of epigenetic asymmetry between parental genomes is that the mammalian oocyte is not truly totipotent, as a paternal genome is also essential for development. Would abolition of epigenetic asymmetry altogether overcome this requirement? Only once in the life of mammals are the parental genomes epigenetically equivalent; following erasure of imprints in primordial germ cells (PGCs). However, transplantation of these imprint-free PGC nuclei into oocytes results in embryonic lethality, partly due to abnormal extraembryonic tissues resulting from the inappropriate silencing or activation of imprinted genes, including loss of function of *Mash2* (*64*). So for imprinting, passage through gametogenesis, especially oogenesis, is essential since a genome devoid of imprints cannot acquire them in a mature oocyte.

Embryos derived after transplantation of the PGC imprint-free nucleus provide insight into the underlying causes of the biallelic expression or repression of imprinted genes. For those genes, such as *Peg3* and *Nnat*, which normally exhibit expression of the paternal allele only, the outcome is biallelic expression. This is because for this category of genes, passage through oogenesis is essential to repress the maternal allele (*64*). In contrast, other genes such as *Igf2r* remain biallelically repressed. These genes require passage through oogenesis to inactivate the antisense transcript through methylation of its promoter, otherwise this transcript predominates in embryos. *H19* that is methylated during spermatogenesis is also biallelically expressed, which results in the loss of expression of *Igf2* (*64*). These latter observations illustrate that epigenetic modifications acquired during spermatogenesis are also critical for appropriate imprinting.

The key questions concerning the erasure of imprints in PGCs, and their initiation during gametogenesis remain unresolved. Recent advances in understanding epigenetic mechanisms may provide essential links between chromatin structure and DNA methylation, and help to fully elucidate the nature of the imprint. Identification of minimal elements in *cis*-control regions, coupled with biochemical and genetic approaches using transgenes may allow systematic unraveling of large clusters of imprinted genes. The availability of genomic sequences from a variety of species will be an increasingly powerful resource, for the detection, discrimination and subsequent analysis of putative control elements. This information is also likely to identify elements that have played a role in the establishment of the imprinting mechanism during evolution, and events that have

Table 1. Relationship between the *Igf2-H19* domain on distal mouse chromosome 7 and the *Dlk1-Gtl2* domain on distal mouse chromosome 12. The striking parallels between the two loci suggest that there may be common features involved in their imprinting control (*31–33*).

5′-*Igf2-H19*-3′	5′-*Dlk1-Gtl2*-3′
1. Reciprocally imprinted genes located 80 to 90 kb apart	Yes
2. 3′ Gene of pair encodes an untranslated RNA	Yes
3. Differential methylation is paternal specific	Yes
4. DMR located in CpG island promoter of 3′ gene	Yes
5. No DMR in CpG island promoter of 5′ gene	Yes
6. 5′ Gene has a DMR in last exon	Yes
7. Intergenic DMR has germ line imprint	Yes
8. Intergenic DMR has CTCF binding sites	No (Conserved putative CTCF-site is intronic)
9. Reciprocal behavior in Dnmt1$^{-/-}$ mouse	Yes

led to the assembly of large imprinted clusters. Although the epigenetic asymmetry between parental genomes remains enigmatic, it appears to have been a vital accompaniment to mammalian evolution, viviparity and placentation, and possibly for the necessary emergence of the trophectoderm lineage for the first time, since it is essential for blastocyst implantation. Consequently, its emergence has had a profound and wide-ranging impact on development in mammals.

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REVIEW

Epigenetic Reprogramming in Mammalian Development

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DNA methylation is a major epigenetic modification of the genome that regulates crucial aspects of its function. Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. However, in mammals there are at least two developmental periods—in germ cells and in preimplantation embryos—in which methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. Epigenetic reprogramming in germ cells is critical for imprinting; reprogramming in early embryos also affects imprinting. Reprogramming is likely to have a crucial role in establishing nuclear totipotency in normal development and in cloned animals, and in the erasure of acquired epigenetic information. A role of reprogramming in stem cell differentiation is also envisaged.

DNA methylation is one of the best-studied epigenetic modifications of DNA in all unicellular and multicellular organisms. In mammals and other vertebrates, methylation occurs predominantly at the symmetrical dinucleotide CpG (*1–4*). Symmetrical methylation and the discovery of a DNA methyltransferase that prefers a hemimethylated substrate, Dnmt1 (*4*), suggested a mechanism by which specific patterns of methylation in the genome could be maintained. Patterns imposed on the genome at defined developmental time points in precursor cells could be maintained by Dnmt1, and would lead to predetermined programs of gene expression during development in descendants of the precursor cells (*5*, *6*). This provided a means to explain how patterns of differentiation could be maintained by populations of cells. In addition, specific demethylation events in differentiated tissues could then lead to further changes in gene expression as needed.

Neat and convincing as this model is, it is still largely unsubstantiated. While effects of methylation on expression of specific genes, particularly imprinted ones (*7*) and some retrotransposons (*8*), have been demonstrated in vivo, it is still unclear whether or not methylation is involved in the control of gene expression during normal development (*9–13*). Although enzymes have been identified that can methylate DNA de novo (Dnmt3a and Dnmt3b) (*14*), it is unknown how specific patterns of methylation are established in the genome. Mechanisms for active demethylation have been suggested, but no enzymes have been identified that carry out this function in vivo (*15–17*). Genomewide alterations in methylation—brought about, for example, by knockouts of the methylase genes—result in embryo lethality or developmental defects, but the basis for abnormal development still remains to be discovered (*7*, *14*). What is clear, however, is that in mammals there are developmental periods of genomewide reprogramming

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