Imprinting evolution and the price of silence

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Summary
In contrast to the biallelic expression of most genes, expression of genes subject to genomic imprinting is monoallelic and based on the sex of the transmitting parent. Possession of only a single active allele can lead to deleterious health consequences in humans. Aberrant expression of imprinted genes, through either genetic or epigenetic alterations, can result in developmental failures, neurodevelopmental and neurobehavioral disorders and cancer. The evolutionary emergence of imprinting occurred in a common ancestor to viviparous mammals after divergence from the egg-laying monotremes. Current evidence indicates that imprinting regulation in metatherian mammals differs from that in eutherian mammals. This suggests that imprinting mechanisms are evolving from those that were established 150 million years ago. Therefore, comparing genomic sequence of imprinted domains from marsupials and eutherians with those of orthologous regions in monotremes offers a potentially powerful bioinformatics approach for identifying novel imprinted genes and their regulatory elements. Such comparative studies will also further our understanding of the molecular evolution and phylogenetic distribution of imprinted genes. *BioEssays* 25:577–588, 2003.

"*It is an error to imagine that evolution signifies a constant tendency to increased perfection. That process undoubtedly involves a constant remodeling of the organism in adaptation to new conditions; but it depends on the nature of those conditions whether the direction of the modifications effected shall be upward or downward.*"

Thomas Henry Huxley
English Biologist/Evolutionist

Mammalian species whose genomes contain imprinted genes are paying a large expense for an obscure genomic modification rooted approximately 150 millions years ago when imprinted genes first arose in ancestral mammals.[1,2] In the case of imprinting, the cost of evolutionary change is associated with the normal silencing of one parental copy of each gene, which depends on the sex of the parent from which it was inherited. This induction of functional haploidy has markedly increased vulnerability to cancer, neurodevelopmental and neurobehavioral disorders, and has implications for the outcomes of assisted reproduction technologies. Imprinting is present in angiosperm plants and eutherian and metatherian mammals. In this review, we will restrict our focus to autosomal imprinting in mammals. We will discuss the current understanding of the molecular foundations of imprinting, disorders associated with imprinted genes, the evolutionary origin of imprinting and the power of phylogenetic comparisons to elucidate imprinted genes and the regulatory elements that determine their unusual pattern of expression.

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Abbreviations: AS, Angelman Syndrome; BWS, Beckwith-Wiedemann Syndrome; DMR, differentially methylated region; IC, imprint center; LOI, loss of imprinting; PGC, primordial germ cell; PWS, Prader-Willi Syndrome; UPD, Uniparental disomy.

Genomic imprinting

We all normally inherit a complete set of chromosomes from each parent, such that for every gene, there is one copy from our mother and one from our father. One correlate of Gregor Mendel's principles of inheritance is that for any given gene and its phenotypic outcome, the sex of the contributing parent is irrelevant. In stark contrast, an estimated 100 to 200 genes within our genomes are subject to genomic imprinting whereby the expression of RNA (coding or non-coding) and protein is a direct consequence of the providing parent's sex. In this case, one parent's copy of the gene is expressed, while the other is silent. The two parental copies of imprinted genes share nearly identical genetic information, yet silencing of one allele necessarily predetermines that any functions ascribed to that gene are now dependent on the single active copy. Over 70 imprinted genes have thus far been identified in mammals (Table 1).

Table 1 Known Imprinted Genes*

	Gene	Human Chromosome	Human	Lemur	Colugo	Tree Shrew	Rat	Mouse	Sheep	Cow	Pig	Opossum	Platypus/ Echidna	Chicken
1)	ARH1	1p31.2	✓	–	–	–	–	–	–	–	–	–	–	–
2)	TP73	1p36.32	✣	–	–	–	–	–	–	–	–	–	–	–
3)	U2AF1RS1	5q22.2	–	–	–	–	–	✓	–	–	–	–	–	–
4)	PLAGL1	6q24.2	✓	–	–	–	–	✓	–	–	–	–	–	–
5)	HYMA1	6q24.2	✓	–	–	–	–	–	–	–	–	–	–	–
6)	SLC22A2	6q25.3	–	–	–	–	–	✓	–	–	–	–	–	–
7)	SLC22A3	6q25.3	–	–	–	–	–	✓	–	–	–	–	–	–
8)	M6P/IGF2R	6q25.3	✗	✗	✗	✗	✓	✓	✓	✓	✓	✓	✗	✗
9)	AIR	Not present	❍	–	–	–	–	✓	–	–	–	–	–	–
10)	GRB10	7p12.2	✓	–	–	–	–	✓	–	–	–	–	–	–
11)	PEG10	7q21.3	✓	–	–	–	–	–	–	–	–	–	–	–
12)	SGCE	7q21.3	–	–	–	–	–	✓	–	–	–	–	–	–
13)	ASB4	7q21.3	–	–	–	–	–	✓	–	–	–	–	–	–
14)	PEG1/MEST	7q32.2	✓	–	–	–	–	✓	✓	–	–	–	–	–
15)	PEG1-AS	7q32.2	✓	–	–	–	–	–	–	–	–	–	–	–
16)	COPG2	7q32.2	✣	–	–	–	–	✓	–	–	–	–	–	–
17)	COPG2IT1	7q32.2	–	–	–	–	–	✓	–	–	–	–	–	–
18)	MIT1/LB9	7q32.2	–	–	–	–	–	✓	–	–	–	–	–	–
19)	WT1	11p13	✣	–	–	–	–	–	–	–	–	–	–	–
20)	H19	11p15.5	✓	–	–	–	✓	✓	✓	–	–	–	–	–
21)	IGF2	11p15.5	✓	–	–	–	✓	✓	✓	–	✓	✓	✗	✗
22)	IGF2AS	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
23)	INS	11p15.5	✓	–	–	–	–	–	–	–	–	–	–	–
24)	INS2	Not present	❍	–	–	–	–	✓	–	–	–	–	–	–
25)	ASCL2	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
26)	TRPM5	11p15.5	✓	–	–	–	–	–	–	–	–	–	–	–
27)	KCNQ1	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
28)	KCNQ1OT1	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
29)	KCNQ1DN	11p15.5	✓	–	–	–	–	–	–	–	–	–	–	–
30)	CDKN1C	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
31)	SLC22A1L	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
32)	TSSC4	11p15.5	✗	–	–	–	–	✓	–	–	–	–	–	–
33)	TSSC3	11p15.5	✓	–	–	–	–	✓	–	–	–	–	–	–
34)	CD81	11p15.5	–	–	–	–	–	✓	–	–	–	–	–	–
35)	ZNF215	11p15.5	✓	–	–	–	–	–	–	–	–	–	–	–
36)	NAPIL4	11p15.5	–	–	–	–	–	✓	–	–	–	–	–	–
37)	MSUIT1	Not present	❍	–	–	–	–	✓	–	–	–	–	–	–
38)	OBPH1	11p15.4	–	–	–	–	–	✓	–	–	–	–	–	–
39)	SDHD	11q23.1	✓	–	–	–	–	–	–	–	–	–	–	–
40)	ATA3	12q13.11	–	–	–	–	–	✓	–	–	–	–	–	–
41)	DCN	12q21.33	–	–	–	–	–	✓	–	–	–	–	–	–
42)	HTR2A	13q14.2	✓	–	–	–	–	✓	–	–	–	–	–	–
43)	MEG3	14q32	✓	–	–	–	–	✓	✓	–	–	–	–	–
44)	DLK1	14q32	✓	–	–	–	–	✓	✓	–	–	–	–	–
45)	DIO3	14q32	–	–	–	–	–	✓	–	–	–	–	–	–
46)	PEG11	14q32	–	–	–	–	–	–	✓	–	–	–	–	–
47)	ANTI-PEG11	14q32	–	–	–	–	–	–	✓	–	–	–	–	–
48)	MEG8/snoRNAs	14q32	–	–	–	–	–	–	✓	–	–	–	–	–
49)	MKRN3	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
50)	ZNF127AS	15q11.2	✓	–	–	–	–	–	–	–	–	–	–	–
51)	NDN	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
52)	MAGEL2	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
53)	SNRPN-SNURF	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
54)	PAR-SN	15q11.2	✓	–	–	–	–	–	–	–	–	–	–	–
55)	HBII-13	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
56)	PWCR1	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
57)	HBII-52	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
58)	IPW	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
59)	PAR1	15q11.2	✓	–	–	–	–	✓	–	–	–	–	–	–
60)	PAR5	15q11-q13	✓	–	–	–	–	✓	–	–	–	–	–	–
61)	PEG12	15q11-q13	–	–	–	–	–	✓	–	–	–	–	–	–
62)	UBE3A	15q12	✓	–	–	–	–	✓	–	–	–	–	–	–
63)	UBE3A-AS	15q12	✓	–	–	–	–	–	–	–	–	–	–	–
64)	ATP10C	15q12	✓	–	–	–	–	–	–	–	–	–	–	–
65)	GABRB3	15q12	✣	–	–	–	–	–	–	–	–	–	–	–
66)	RASGRF1	15q25.1	–	–	–	–	–	✓	–	–	–	–	–	–
67)	ELONGIN A3	18q21.1	✓	–	–	–	–	–	–	–	–	–	–	–
68)	IMPACT	18q11.2-q12.1	✗	–	–	–	–	✓	–	–	–	–	–	–
69)	PEG3/ZIM2	19q13.43	✓	–	–	–	–	✓	–	–	–	–	–	–
70)	ZIM1	Not present	❍	–	–	–	–	✓	–	–	–	–	–	–
71)	USP29	19q13.43	–	–	–	–	–	✓	–	–	–	–	–	–
72)	ZIM3	19q13.43	–	–	–	–	–	✓	–	–	–	–	–	–
73)	ZNF264	19q13.43	–	–	–	–	–	✓	–	–	–	–	–	–
74)	NNAT	20q11.23	✓	–	–	–	–	✓	–	–	–	–	–	–
75)	GNAS1	20q13.32	✓	–	–	–	–	✓	–	–	–	–	–	–
76)	GNAS1-AS	20q13.32	✓	–	–	–	–	✓	–	–	–	–	–	–

✓ Imprinted ✣ Imprint status disputed – Imprint status unknown ✗ Not imprinted ❍ Gene not present

* Data adapted from http://www.otago.ac.nz/IGC

Imprint erasure, establishment and maintenance

Because the parental genomes are each contained in separate physical compartments only in gametes, these cells must contain information that distinguishes the "sex" of imprinted genes for appropriate expression in the next generation. These marks, which differ within the offspring on each of the two inherited sister chromatids, must be erased in the germ cells of each generation and then re-established such that the profile of these cells reflects the sex of the individual in which they reside. Once the imprints are founded, these markings must also be maintained in somatic cells throughout all subsequent cellular divisions so that the transcription machinery can appropriately interpret the information to effect accurate expression. This dynamic process is complex and involves erasure, establishment, maintenance and implementation of the imprint markings.

DNA methylation and imprint marks

Other than typical sequence polymorphisms that do not correlate with imprint status, the nucleotide sequence of the two alleles of imprinted genes are identical. Therefore, the imprint marks that distinguish the two parental alleles must be epigenetic in nature. Epigenetic alterations are defined as modifications that induce heritable changes in gene expression without changes in DNA sequence. Chromatin structure is an example of an epigenetic characteristic, and is regulated by a number of factors including histone methylation, histone acetylation and cytosine methylation. Cytosine methylation is a vital DNA modification[3] that is important for the regulation of many aspects of cellular function including imprinting.[4,5] The 5-carbon position of cytosines in the context of CpG dinucleotides throughout the mammalian genome is subject to the covalent attachment of a methyl group. Unlike cytosine, 5-methylcytosine is highly susceptible to spontaneous deamination that results in the generation of a TpG dinucleotide. Consequently, the mammalian genome has become progressively depleted of CpG dinucleotides through the course of evolution. However, there are genomic regions ranging from several hundred to several thousand base pairs in length that have maintained the expected number of CpGs. Because they are normally unmethylated, these "CpG islands" have presumably been protected from such spontaneous deamination. CpG islands are found associated with the promoter regions of roughly 40–50% of housekeeping genes. Important exceptions to the unmethylated status of CpG islands include those that are associated with imprinted genes, genes subject to X chromosome inactivation and transposable elements. Pathological CpG island hypermethylation is well documented for many genes involved in cancer.[6] Methylation of the two parental alleles is not equivalent for imprinted genes, and sequences exhibiting such contrasting epigenetic characteristics are referred to as "differentially methylated regions", or DMRs.

Erasure and establishment

The selective silencing of imprinted genes is modulated with the life cycle of the organism (Fig. 1). Each gamete carries sex-specific imprint markings that are required for normal development.[7] Upon fertilization, the two epigenetically distinct pronuclei exist in the same cellular environment, dominated by oocyte-specific factors. Remarkably, the paternal pronucleus is rapidly and actively demethylated within the zygote prior to the first cellular division.[8] In contrast, the maternal genome becomes demethylated in a passive manner during subsequent divisions, presumably due to a lack of methyltransferase activity on the hemimethylated DNA substrate. Erasure of the paternal methylation profile by the oocyte is a potentially powerful mechanism by which maternal factors modify chromatin structure to regulate the paternal genome.[9] Demethylation at this stage in development is proposed to be required for the activation of genes necessary for early embryonic growth.[10] However, imprint methylation marks present on both the paternal and maternal genomes are maintained despite this global demethylation event.[8,11]

Another reprogramming event occurs later, within the primordial germ cells (PGCs) of the developing fetus. Between 10.5 and 12.5 days post-coitum in mice, when the developing germ cells are entering the gonads, there is an apparently complete eradication of DNA methylation (with the exception of multicopy repeat sequences).[12] After demethylation in the PGCs, parental-specific methylation is re-established during gametogenesis (Fig. 1). This occurs in sperm postnatally within diploid gonocytes prior to meiosis and within oocytes arrested at the diplotene stage of meiosis.[13] A testes-specific zinc finger DNA-binding protein was recently identified that may play a pivotal role in this process. BORIS (Brother Of the Regulator of Imprinted Sites), a paralog of the CCCTC-binding factor protein, CTCF, functions in the implementation of imprinting (see below) to control parental-specific expression.[14] Despite divergent amino and carboxy termini, both BORIS and CTCF bind to the same DNA sequences by virtue of each having identical zinc finger domains, likely the result of a gene duplication event. However, their expression is mutually exclusive, suggesting that they carry out similar functions that are subject to strict spatiotemporal regulation.

BORIS expression in testes is normally limited to the discrete period of spermatogenesis when methylation patterns are erased. This has led to the intriguing hypothesis that BORIS may facilitate de novo establishment of the methylation imprints, while CTCF functions to maintain and/or read these imprint marks pre- and postnatally.[14,15] Supporting this role, a link between CTCF and the protein complexes required for establishing chromatin silencing through histone

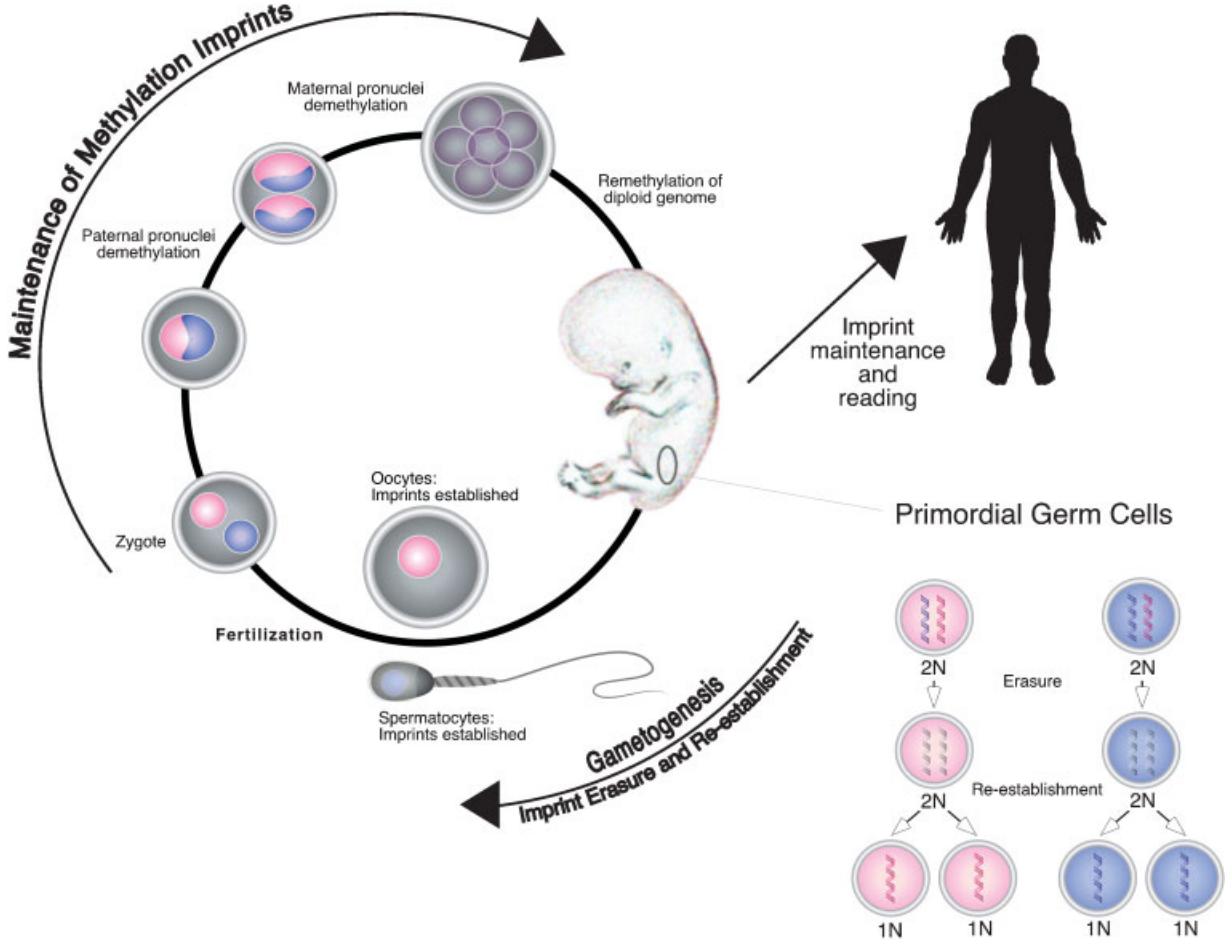

Figure 1. Imprinting throughout development. Methylation marks associated with imprinted genes are established at two distinct phases of development. During gametogenesis, the imprint marks present on the maternal (pink) and paternal (blue) chromosomes are erased (grey), followed by establishment of primary imprints which then reflect only the sex of the individual in which they reside. Just after fertilization, a global demethylation event occurs in the zygote, first in the paternal pronucleus (blue), followed by the maternal pronucleus (pink). Imprint marks that were established in the gametes must resist this demethylation process. Remethylation of the diploid genome occurs post-implantation, and includes setting of the secondary imprints, which are maintained throughout the lifespan of the individual.

acetylation has been demonstrated.[16] Furthermore, colocalization of CTCF and BORIS with the histone methylases Suv39h1 and Suv39h2[15] suggests a connection between these proteins and the prerequisite methylation of histones for directing de novo DNA methylation.[17]

The DNA features that direct acquisition of methylation imprints are not clear. Consensus CTCF/BORIS binding sites are located at or near the paternal germline methylation imprint marks associated with the *Igf2*/*H19* and *Dlk1*/*Meg3* regions (see below). Since CTCF/BORIS binding is regulated in a combinatorial fashion via multiple zinc finger domains, there may be other as yet undefined sequence binding motifs associated with other imprinted genes. In addition, these imprinted genes and many others (e.g., *M6p*/*Igf2r*, *Peg1*/*Mest*, *Impact* and *Rasgrf1*) are associated with tandem repeat sequences in close proximity to germline methylation imprint marks, and these repeats may function in coordinating the methylation profile. For *Rasgrf1*, tandem repeats upstream from the promoter are necessary for the correct establishment of methylation at an adjacent CpG island in sperm and subsequent imprinted expression.[18] Although histone acetylation patterns that appear to be specific to parental origin have also been identified, less is known about this aspect of chromatin structure in the establishment of germline imprints. It is unlikely to mediate heritability of paternal epigenetic information, however, since sperm chromatin is devoid of histones and instead consists of DNA wrapped together with protamines.[19]

In the oocyte, the Dnmt3 family of methyltransferases is required to set maternal-specific methylation patterns

for imprinted genes. The Dnmt3 family member, Dnmt3L is essential, yet lacks methyltransferase activity.[5] Dnmt3L is therefore proposed to provide sequence specificity for the de novo methyltransferases, Dnmt3a and Dnmt3b, by directing them to the DNA regions requiring maternal methylation imprints.[20] These findings in mice have been underscored by the report of a human female whose fertilized oocytes are devoid of all normal maternal methylation marks at imprinted loci. This condition, which suggests a deficit in DNMT3-mediated imprint marking, leads to post-implantation lethality.[21] In addition to their role in establishing methylation patterns in oocytes, Dnmt3a, Dnmt3b and Dnmt3L can recruit histone deacetylases, which are thought to synergize with Dnmts in the initiation of gene silencing.[22] Dnmt3 proteins are also required for spermatogenesis,[20] but it is not known whether they provide the de novo methylation required to establish a paternal imprint mark.

Maintenance

Once specific patterns of CpG methylation are re-established in the somatic cells within the developing embryo, they must be faithfully maintained throughout many rounds of DNA replication during growth and development. This is accomplished through the actions of maintenance methyltransferases such as DNMT1, which recognizes hemimethylated CpG sites at replication foci and adds methyl groups to cytosines on the nascent DNA strand to replicate the methylation pattern of the parent strand. DNA replication is intimately associated with establishment of chromatin structure, which involves concomitant incorporation of histone proteins to form nucleosomes. Newly synthesized histones arrive at replication foci in an acetylated form; typically, acetylated histones are associated with transcriptionally active (euchromatic) genomic regions. To perpetuate transcriptionally inactive (heterochromatic) regions, such as pericentric chromosomal regions and the silenced allele of an imprinted gene, histones must be enzymatically deacetylated. DNMT1 has been shown to associate with histone deacetylases at replication foci,[23] combining DNA synthesis with the replication of chromatin structure to produce a genetically and epigenetically identical daughter cell. Whereas histone acetylation apparently does not function as a germline imprint mark (see above), this chromatin modification may have an important role in the maintenance of proper imprinted gene expression. Imprinted genes in somatic tissues indeed exhibit parent-of-origin-specific histone acetylation patterns that presumably contribute to regulating the transcriptional activity of the maternally and paternally derived alleles.[24,25] Therefore, maintenance of the chromatin conformation associated with imprinted genes likely requires DNA methylation coupled with histone acetylation. The importance of the proper regulation of imprinting throughout the lifespan of an individual is evidenced by the observation that defects in erasure, establishment and maintenance of imprint marks are all associated with a wide variety of disorders and diseases in humans.

The price of imprinting

Parental-specific gene expression has a deleterious consequence in that functional haploidy eliminates the protection that diploidy normally provides against recessive mutations. Moreover, the complex epigenetic mechanisms that regulate monoallelic expression of imprinted genes are susceptible to dysregulation at multiple levels. Accordingly, imprinted regions of the genome are associated with a number of diverse developmental disorders and diseases that result from impaired regulation, altered dosage or mutation of these domains. Since imprinted genes often occur in clusters coordinately regulated by imprint control centers, single genetic or epigenetic alterations in these key regions can lead to disruption of many genes resulting in the formation of multiple disorders.

Imprinting and uniparental disomy

Uniparental disomies (UPDs) result when an individual inherits two copies of the same chromosome or subchromosomal region from only one parent. The karyotype of these individuals appears normal since they are diploid, but because of the uniparental chromosome inheritance, they can exhibit problems not only with non-imprinted genes when recessive mutations are exposed, but also for any imprinted genes within the disomic region due to doubling or absence of expression. This has led to the identification of multiple syndromes that result from global disruption of imprinting on the disomic chromosomes. For example, paternal UPD for chromosome 6 [upd(6)pat] is associated with transient neonatal diabetes, upd(7)mat with Silver-Russell syndrome, upd(14)mat with intrauterine growth retardation and precocious puberty, and upd(14)pat with growth retardation, small thorax and mental retardation. Prader-Willi and Angelman syndrome result from maternal and paternal chromosome 15 duplications, respectively, and Beckwith-Wiedemann syndrome occurs with upd(11)pat (see below). Other UPD-related phenotypes suspected of having an imprinting effect include growth failure and bronchopulmonary dysplasia found in individuals with upd(2)mat and low birth weight and congenital malformations in individuals with upd(16)mat.[26]

Interestingly, no individuals with UPD have been reported for chromosomes 18 and 19,[26] each of which has at least one imprinted gene (Fig. 2). This may indicate that aberrant imprinted gene expression from these chromosomes results in a nonviable pregnancy. This is supported by the observation that mice with maternal duplication in the region of mouse chromosome 7 syntenic with the imprinted region on human chromosome 19q do not survive.[27]

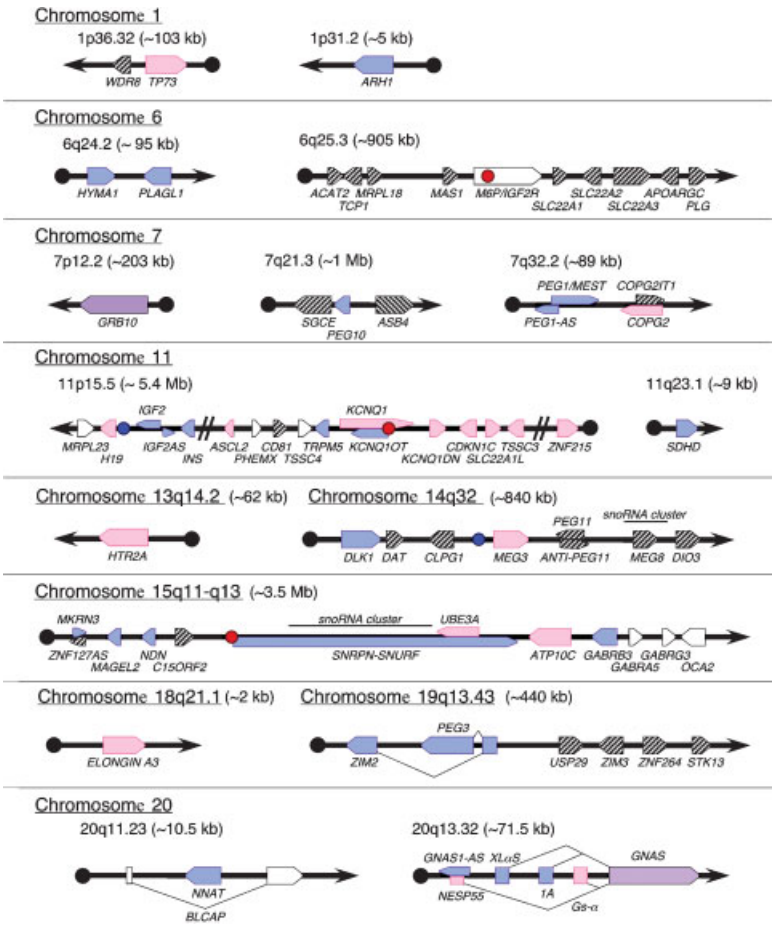

Figure 2. The known human imprinted gene clusters; not to scale. For detailed information see http://www.otago.ac.nz/IGC, http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html and http://www.geneimprint.com. The chromosomal location is indicated along with the approximate size of each region. Pink, maternally expressed gene; blue, paternally expressed gene; hatched, imprint status not yet examined in humans; purple, maternal and paternal transcripts distinctly expressed depending on promoter usage or alternative splicing; white, biallelic expression. Blue circles, paternally methylated imprint center (IC); red circles, maternally methylated IC. Direction of transcription is indicated. Arrows point toward the telomere for each domain.

Imprinting and cancer

The role of imprinted genes in growth-related pathways is consistent with the observation that genomic imprinting is involved in many types of cancers. Numerous imprinted genes show abnormal changes in gene expression and alterations in the normal pattern of methylation in cancer. One of the most common findings has been a ''loss of imprinting'' (LOI), which refers to epigenetic modifications that result in either the activation of gene expression on the normally silenced allele or the loss of expression from the normally active allele. An intensively studied example is *IGF2*, which exhibits LOI in numerous childhood and adult cancers (for reviews, see Refs. 28,29). *IGF2* imprinting abnormalities are implicated in the pathogenesis of cancers associated with Beckwith-Wiedemann syndrome, including Wilms' tumor, hepatoblastomas, rhabdomyosarcoma, and adrenal carcinoma. The mechanisms underlying biallelic expression of *IGF2* are diverse, but can involve alternate promoter usage for *IGF2*, alterations in the *KvLQT1-AS* imprinting center (IC1; the heritable gametic imprint mark) in the centromeric part of the imprinted domain, and methylation changes in the germline DMR (IC2) upstream of *H19* (Fig. 2). LOI is correlated with hypermethylation of the CTCF-binding sites (and consequent loss of boundary function) within IC2 on the maternal chromosome in colorectal cancer and Wilms' tumor.[30,31]

Tumor cells appear to utilize multiple mechanisms to maintain elevated levels of IGF2. The M6P/IGF2R is a multifunctional receptor that binds to IGF2 and transports it to the lysosomes where it is degraded, therefore playing a critical role in restraining IGF2-mediated cell growth. Not surprisingly, the *M6P*/*IGF2R* is subject to loss of heterozygosity in a number of tumors[32–36] which is frequently accompanied by inactivation of the remaining allele by mutations in the IGF2-binding domain.[32] The growth advantage obtained by *M6P*/*IGF2R* inactivation would be further promoted in tumors that also have increased *IGF2* expression because of LOI; however, this potential combination of pathological alterations has not yet been examined.

Several other imprinted genes are implicated in tumor formation by virtue of loss or gain of expression. These include

genes with proposed tumor suppressive functions (*ARH1*, breast and ovarian cancer; *PEG3*, gliomas; $p57^{KIP2}$, Wilms' tumor; *ZAC*, breast cancer; and *NNAT*, acute myeloid leukemia) and those with tumor-promoting functions (*PEG1*/*MEST*, invasive breast cancer, uterine leiomyoma and lung cancer; and *DLK1*, uterine leiomyoma, neuroendocrine tumors). Unlike genetic mutations, the reversible nature of epigenetic lesions may render them amenable to therapeutic intervention. For example, it may be possible to use inhibitory drugs that target the enzymes responsible for DNA methylation and histone deacetylation such as the DNMT inhibitors, 5-azacytidine and 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor, trichostatin A. DNMT inhibitors in fact are efficacious in treating cancer.[37] This exciting potential for treatment underscores the critical need for further studies on the mechanisms by which imprinted genes contribute to tumor development.

Imprinting and neurobehavioral and developmental disorders

Several neurogenetic and developmental disorders, including Angelman, Prader-Willi, and Beckwith-Wiedemann syndromes are associated with specific imprinted regions and genes.[26,38] Prader-Willi syndrome (PWS) is associated with loss of paternal gene expression at chromosome 15q11-q13. Affected infants present with hypotonicity and failure to thrive. Later in development, they exhibit hyperphagia and can become severely obese. These individuals also have short stature, mild mental retardation and obsessive compulsive disorder. Angelman syndrome (AS) results either from loss of maternal gene expression of the same region of chromosome 15 or from mutations in *UBE3A*. Affected individuals exhibit ataxia, severe motor and mental retardation, lack of speech, epilepsy and hypotonia (see Refs. 38,39 for recent reviews). Beckwith-Wiedemann syndrome (BWS) is characterized by a generalized disruption of imprinting at chromosome 11p15.5 with exomphalos, macroglossia, somatic overgrowth, and predisposition to multiple types of cancer.[40] In all three of these imprinting disorders, epigenetic alterations have an important contributory or causative role.

A possible etiologic contribution of imprinted genes is suggested by other disorders that exhibit parent-of-origin effects. These include Silver-Russell syndrome (maternal; chromosome 7); bipolar disorder (paternal; chromosome 18), schizophrenia (paternal; chromosome 22), Tourette's syndrome (maternal; location unknown) and autism (maternal, chromosome 15; and paternal, chromosome 7). Evidence of a specific gene's involvement in most of these disorders has not been forthcoming, however, even with extensive mutational analyses of genes present within the areas of linkage association. This may be due to the proposed multigenic nature of these complex disorders in that the ability to correlate a distinct genetic alteration with its presence within the affected population may be diluted by the contribution of other involved genes. Alternatively, the lack of genetic alterations in candidate genes residing in the genomic regions with linkage association may indicate the involvement of epigenetic, rather than genetic, alterations. The breadth of known epigenetic alterations in imprinted genes for BWS, AS and PWS as well as cancer underscores the need to evaluate these other developmental and cognitive disorders in this same light.

For example, it is plausible that an individual's epigenetic profile, which is heritable and tissue-specific, is composed of ''epihaplotypes'' (see also Ref. 41) just as the DNA sequence itself is organized into haplotypes based on genetic variation. We define epihaplotypes as the aggregation of epigenetic characteristics (e.g., CpG methylation and histone acetylation patterns) for a closely linked group of alleles on the same chromosome. We postulate that epigenetic and genetic variability combine to contribute to many of the neurogenetic disorders with parent-of-origin effects.

How might these epigenetic alterations be induced? One possibility is the positioning of normally methylated transposable elements in the vicinity of unmethylated CpG islands that regulate gene expression. This type of genetic fingerprint has already been shown to dramatically influence phenotype in the mouse *Agouti* gene, due to a retroviral element insertion that promotes epigenetic instability and ectopic expression, depending on the degree of methylation.[42] Given the transposon load carried in the human genome combined with the exclusion of some of these elements from imprinted domains in primates and rodents,[43,44] the potential for detrimental effects as a result of transposon-gene adjacency requires further investigation.

Importantly, epihaplotypes represent the consequence of a direct interface between environmental influences and gene regulation. Methylation (and presumably histone acetylation) can be altered depending on environmental conditions at the cellular and organismic levels, especially during early development when the methylation patterns are established.[45–47] For example, epigenetic alterations are evident from in vitro culture of embryonic stem cells[48] and preimplantation embryos.[49] Indeed, the majority of cloned embryos exhibit abnormal methylation patterns,[50] and demethylation of DMR2 in the *M6P*/*IGF2R*, with subsequent loss of gene function, is highly associated with ''large offspring syndrome''.[51]

Another possible explanation for the inability to correlate neurogenetic disorders having parent-of-origin effects with a specific gene mutation is that the causative mutation may reside in a previously overlooked chromosomal location. For example, introns and intergenic regions may contain sequences that are pertinent to regulating a gene(s) whose dysregulation ultimately gives rise to the abnormal phenotype. In this regard, comparative genomic approaches will be

invaluable in defining evolutionarily conserved and thus biologically important regions to evaluate for genetic or epigenetic alterations.

Imprinting and behavior

Several imprinted genes are suggested to contribute to the ability of female mice to nurture their pups, one of the most fundamental of mammalian behaviors. For the paternally expressed genes *Peg1*/*Mest*[52] and *Peg3*,[53] possession of a nonfunctional paternal allele in parturient females results in apathy toward nest building, lack of pup retrieval, inefficient pup nursing and, unlike the majority of eutherian mammals, lack of afterbirth ingestion (for review, see Ref. 54). Most pups born to mutant mothers do not survive; those that do survive are runted. The maternal deficit in nurturing behavior is of direct consequence to offspring of a daughter who receives the defective allele from her father. Studies in rats have shown that *Peg3* and *Peg5*/*Nnat* are both highly expressed in the vasopressin-positive magnocellular neurons during lactation; *Peg3* is also expressed in the oxytocin-positive magnocellular neurons.[55] In parturient female mice with a defective paternal copy of Peg3, there is also a marked decrease in oxytocin-positive neurons and impaired milk ejection, indicative of an important role for this imprinted genes in lactation.[53] The potential contribution of Peg5/Nnat to maternal nurturing behavior has not yet been examined.

In addition to the maternal effects on pup growth and survival, offspring directly inheriting a defective paternal copy of *Peg1*/*Mest* or *Peg3* are also growth impaired.[54] For both *Peg1*/*Mest* and *Peg3*$^{+/-}$ offspring derived from crosses of $+/-$ males to $+/+$ females, a 20% decrease in size at birth was observed that increased to 35% by one week postpartum for *Peg1*/*Mest* mutants and four weeks postpartum for *Peg3* mutants. This indicates a significant defect in the ability of mutant pups to extract adequate resources from their wild-type mother both pre- and postnatally as a consequence of a defective paternally expressed gene.

PEG1/*MEST*, *PEG3*, and *PEG5*/*NNAT* are also imprinted in humans[56–58] with 90% nucleotide identity between mouse and human for *PEG1*/*MEST*, 83% identity for *PEG3*, and 89% identity between rats and humans for *PEG5*/*NNAT*. This strong level of nucleotide conservation is indicative of a shared biological function. It remains unknown, however, whether the behavioral effects of *Peg1*/*Mest* and *Peg3* extend to non-rodent mammals.

Imprinting and assisted reproduction

Attention has recently been focused on the role of imprinting in the outcome of in vitro fertilizations, including those assisted by intracytoplasmic sperm injection. Concern has been raised over potential problems associated with abnormalities in epigenetic reprogramming that can occur during in vitro oocyte maturation, fertilization and zygote culture prior to implantation. This is a critical time for the proper establishment of the epigenetic characteristics that define chromatin structure (Fig. 1), and the egg and the zygote may therefore be especially vulnerable to perturbations due to non-physiological environmental conditions encountered in vitro. Such epigenetic defects have already been observed in other species.[51,59,60]

Several recent reports have documented a troubling increased incidence of imprinting abnormalities in children conceived through assisted reproductive technology. These include reports of three children with AS with rare sporadic imprinting defects on chromosome 15q11-q13[61,62] and eleven children with BWS.[63,64] This represents an approximate 3- to 6-fold increased risk for BWS in individuals conceived through assisted reproduction techniques compared to the incidence of BWS in the general population.

These reports strongly suggest a need for intensive investigation into the aspects of assisted reproductive technologies that contribute to these types of imprinting defects. Whether other types of imprinting abnormalities are associated with in vitro fertilizations in humans is currently unknown. Given that this technique has been successfully performed only since 1978, it may be too early to determine if individuals that have been conceived in this manner are at heightened risk as adults for cancers associated with imprinting abnormalities.

Evolution of imprinting

Imprinted genes have thus far been identified in metatherian (marsupial) and eutherian (placental) mammals[1,65] but not in prototherian (monotreme) mammals.[1] Multiple theories have been proposed to explain the origins of imprinting early in mammalian evolution [e.g., see Refs. 9,66]. According to the most debated of these theories, the "conflict hypothesis",[67] the genetic contention between the male and female genomes is predicted to occur at the materno-fetal interface. This portion of development positions the offspring to extract nourishment directly from the mother; during this time the father's genes have the opportunity to influence the growth and competitive fitness of his offspring within the uterine environment. The mother's genes are also capable of regulating energy distribution through the placenta to the offspring. By this rationale, imprinting of genes would only be relevant to mammalian species with intrauterine gestation, and should be absent in egg-laying species. For several genes examined so far, imprinting is indeed absent in both monotremes (egg-laying mammals) and avian species, including the chicken.[65,68] Imprinting is present in the marsupial opossum,[1,65] which has an 11–13 day intrauterine gestation. Although non-invasive, the opossum placenta functions during a short period (approximately three days) in which there is nutrient transfer via the maternal circulation to the developing embryos.[69,70] Marsupial fetal development is carried out ex utero, so imprinting may also be evident for genes that influence lactation

(e.g., *Peg3* and *Peg5*/*Nnat*, Refs. 53,55) and postnatal nurturing behavior (e.g., *Peg1*/*Mest*, Ref. 52 and *Peg3*, Ref. 53) in addition to placental nutrient transfer. It will be interesting to determine if genes that influence such postnatal behaviors are imprinted in monotremes.

Evolution of IGF2 and M6P/IGF2R imprinting

Many imprinted genes have essential roles in fetal growth and development. The best characterized of these were among the first imprinted genes identified: the maternally expressed *mannose-6-phosphate/insulin-like growth factor 2 receptor* (*M6p*/*Igf2r*) and paternally expressed *insulin-like growth factor 2* (*Igf2*). The *cation-independent mannose 6-phosphate receptor* (*M6pr*) encodes a receptor molecule involved in lysosomal trafficking of proteins with mannose 6-phosphate moieties. This receptor also acquired the ability to bind Igf2 sometime between the evolutionary divergence of the therian from prototherian mammals.[1] Interestingly, the *M6p*/*Igf2r* also acquired its imprinted status at this time, indicating that the ability to bind Igf2 perhaps contributed a selective force for the father to inactivate his copy of this gene.[71] Igf2 is a highly conserved, potent mitogen that stimulates placental and fetal growth in utero.[72] Igf2 serves as a ligand for the Igf1 receptor and the insulin type A receptor. This binding functions to initiate and propagate growth-inducing signals and block apoptosis. In contrast, binding of Igf2 to the M6p/Igf2r neutralizes this growth factor by trafficking it into the lysosomes for degradation. The reciprocal imprinting and common biological pathway for Igf2 and the M6p/Igf2r has led to much speculation about the origin, evolution, and biologic rationale behind imprinting of these two genes.

Because *Igf2* is imprinted in marsupials but not in monotremes, imprinting of this gene must have originated in ancestors of the Therian mammals approximately 150 million years ago. In humans and mice, the heritable imprint methylation mark for *Igf2* is carried on the paternal chromosome, just upstream from the maternally expressed *H19*. It is unclear whether the imprint machinery visible in species today is the same as that used to initially imprint ancestral genes. Experimental evidence demonstrates that the mechanism used for *M6p*/*Igf2r* imprinting in the opossum is different from that in mice.[1] Mice have two DMRs associated with the *M6p*/*Igf2r*: DMR1 spans the promoter region and is paternally methylated and DMR2 is located within intron 2 of the *M6p*/*Igf2r* and carries a heritable maternal methylation imprint.[73] Both DMRs are required for proper imprinted expression of *M6p*/*Igf2r* in mice.[74] Interestingly, humans share the same DMR2 methylation profile for *M6p*/*Igf2r* yet exhibit biallelic expression of this locus in all tissues and developmental stages tested thus far.[75] Furthermore, *M6P*/*IGF2R* is imprinted in the marsupial opossum despite its complete lack of a DMR2.[1] The details of the mechanisms used by the opossum to regulate imprinting of the *M6P*/*IGF2R* remain unclear. The lack of DMR2 in this species, combined with the loss of imprinted *M6P*/*IGF2R* expression in humans, is indicative of selective forces that first established the imprint but then caused it to be lost.

Theoretically, the consequence of loss of imprinted status for *M6P*/*IGF2R* in primates and their nearest non-primate relatives combined with retention of *IGF2* imprinting is a doubling of the expression ratio of growth-suppressing M6P/IGF2R to growth promoting IGF2. The simplest explanation for the evolutionary alteration in *M6P*/*IGF2R* imprint status would be a selective pressure that favored the ability to further decrease biological function of IGF2. Since IGF2 serves a mitogenic function, restoration of biallelic expression for the *M6P*/*IGF2R* may have helped to effectively reduce the concentration of circulating IGF2 leading to a reduction in overall size. Indeed, mice expressing both *M6p*/*Igf2r* alleles exhibit a 20% decrease in body size late in development that persists into adulthood.[76]

Evolutionary studies indicate a rapid shift toward increasing brain size in species with increasingly complex social structures.[54] Difficulty in parturition and maternal perinatal morbidity and mortality might have initially accompanied this anatomical modification. Therefore, one possibility for the recent loss of *M6P*/*IGF2R* imprinting in an ancestor of the near-primates and primates might be that this allowed for increased maternal survival during birthing by effectively reducing body and/or brain size. Alternatively, the imprinted status of the *M6p*/*Igf2r* may predispose to tumor formation,[77] and acquisition of biallelic expression may have provided an evolutionary advantage by reducing cancer incidence in individuals through reproductive age. It remains unclear why a loss of imprinting for *M6P*/*IGF2R* was favored in this instance as opposed to an increase in expression from the maternal allele.

Future focus: comparative sequencing

The analysis of genomic sequence information from different mammalian species has enabled revealing comparisons of imprinted domains. Included in these studies are comparisons of the chromosome 11 imprinted domain in human, mouse and pig[78–81] and the *DLK1*/*MEG3* imprinted domain in human, mouse and sheep.[82] In addition to determining that imprinted gene structure and sequence are conserved, these studies also identified a number of novel genes.[79,82,83] Non-exonic sequence elements and tandem repeats[82] were also conserved, and similarities and differences between the size, distribution and density of CpG islands were observed.[84] The tremendous power in such comparisons is highlighted by the recent demonstration that they can reveal elusive *cis*-acting control elements associated with establishment, maintenance or regulation of imprinting.

For example, phylogenetic comparisons were instrumental in the recent characterization of the mutation causing the

"callipyge" phenotype in sheep.[85] Callipyge sheep are characterized by fast-twitch muscular hypertrophy with concomitant decrease in adiposity in the affected muscles accompanied by a 30% increase in feed efficiency. The phenotype is most apparent in longissimus dorsi muscle; this led to the provocative description of the affected sheep as "callipyge" (Greek: calli-, beautiful; -pyge, buttocks). A role for imprinting was evident from the inheritance pattern of the phenotype; callipyge animals result only when the trait is inherited from their sire and the dam contributes a nonaffected allele. Interestingly, homozygous callipyge offspring are of normal phenotype, which led to the description of this unusual inheritance pattern as "polar overdominance".[86]

The callipyge mutation was mapped to ovine chromosome 18 in a region orthologous to chromosome 14 in humans and chromosome 12 in mice that contained the imprinted genes *DLK1* and *MEG3*.[87,88] Sequence analysis of these and other candidate genes in the linkage interval failed to identify mutations. Further sequencing of the entire linkage region using sheep identical-by-descent to the founder animal revealed a single transition mutation that perfectly correlated with the callipyge phenotype.[85] This mutation was not in a previously recognized gene or regulatory region. However, comparison of human, mouse, bovine, and ovine sequence showed that a 144 bp region encompassing the mutation was highly conserved. Further investigation led to the identification of a novel transcript produced from the region containing the mutation, lending credence to the power of comparative genomics to identify otherwise elusive regulatory elements and genes.

Based on studies of *IGF2* and *M6P/IGF2R*,[1,2,75] the divergence in imprinting among extant prototherian, metatherian and eutherian mammals may offer an unprecedented opportunity to identify important regulatory and/or genomic features of imprinted domains. Comparisons of these regions in the nonimprinted monotremes with those in the imprinted marsupials is expected to reveal the acquisition by marsupials of genomic or epigenetic features relevant to the establishment of imprinting, such as CpG islands, tandem repeat elements, noncoding RNAs, and perhaps other as yet undefined features. Further comparisons of the marsupial genome to the genomes of rodents and primates should also reveal specific features of imprinted domains that either have maintained the status quo throughout evolution or have evolved further due to ongoing selective processes.

Such differences have already been demonstrated for the imprinted opossum *M6P/IGF2R*, which lacks the DMR2 known to be required in all other imprinted species examined to date.[75] We postulate that comparative genomic studies of imprinted domains within the three mammalian subclasses will prove to be a powerful approach to mapping imprinted domains. Previous comparisons between rodents, primates, and artiodactyls have revealed extensive areas of conservation such that critical imprinting elements are difficult to distinguish from the sequences conserved due to the relatively close phylogenetic relationships between these species. In contrast, the increased evolutionary distance between the monotremes, marsupials and primates coupled with the divergent imprinting between these groups should significantly improve the ability to refine extraction of the biologically relevant regions involved in imprinting.

Other more global questions about the origins of imprinting mechanisms may have answers that will be revealed through examination of the monotremes. For instance, if further studies substantiate that the monotremes lack genomic imprinting entirely, it will be of interest to determine whether the monotremes also lack the machinery involved in the establishment of imprinting, such as the testis-specific BORIS protein and the DNMTs involved in imprint establishment. Alternatively, perhaps sequence characteristics, such as an abundance of repetitive elements in the vicinity of the relevant genes, has hindered the ability to establish differential chromatin structure in the monotremes in spite of the presence of the essential machinery. Further examination of the monotreme and marsupial genomes should provide great insight into this unique phenomenon.

Conclusions

This review has focused on the mechanisms underlying genomic imprinting, its deleterious consequences, and the evolution of this unique form of gene regulation. The "price of silence" due to imprinting takes a tremendous toll not only in terms of societal costs of treating imprinting disorders, but more importantly in human health and the well being of affected individuals and their families. We are now only beginning to understand the contribution of imprinted genes to human morbidity and mortality with many more imprinted genes yet to be identified. A more comprehensive understanding of these genes and their regulation will undoubtedly unfold in the coming years. This will not only further our knowledge of the fundamental roles imprinted genes play in mammalian development, but also will likely lead to the discovery of novel therapeutic approaches to treat the many disorders of imprinting.

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