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The origin and evolution of genomic imprinting and viviparity in mammals

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Genomic imprinting is widespread in eutherian mammals. Marsupial mammals also have genomic imprinting, but in fewer loci. It has long been thought that genomic imprinting is somehow related to placentation and/or viviparity in mammals, although neither is restricted to mammals. Most imprinted genes are expressed in the placenta. There is no evidence for genomic imprinting in the egg-laying monotreme mammals, despite their short-lived placenta that transfers nutrients from mother to embryo. Post natal genomic imprinting also occurs, especially in the brain. However, little attention has been paid to the primary source of nutrition in the neonate in all mammals, the mammary gland. Differentially methylated regions (DMRs) play an important role as imprinting control centres in each imprinted region which usually comprises both paternally and maternally expressed genes (*PEGs* and *MEGs*). The DMR is established in the male or female germline (the *gDMR*). Comprehensive comparative genome studies demonstrated that two imprinted regions, *PEG10* and *IGF2-H19*, are conserved in both marsupials and eutherians and that *PEG10* and *H19* DMRs emerged in the therian ancestor at least 160 Ma, indicating the ancestral origin of genomic imprinting during therian mammal evolution. Importantly, these regions are known to be deeply involved in placental and embryonic growth. It appears that most maternal *gDMRs* are always associated with imprinting in eutherian mammals, but emerged at differing times during mammalian evolution. Thus, genomic imprinting could evolve from a defence mechanism against transposable elements that depended on DNA methylation established in germ cells.

1. Introduction

Parent-of-origin gene expression (genomic imprinting) is widespread amongst eutherian mammals and also occurs in marsupials. Most imprinted genes are expressed in the placenta, but the brain is also a favoured site. Although imprinting evolved in therian mammals before the marsupial–eutherian split, the mechanisms have continued to evolve in each lineage to produce the differences in the number and regulation of imprinted genes that now exist between the two groups. There are around 100 genes that are subject to genomic imprinting in eutherian mammals, but there appears to be many fewer in marsupial mammals. Marsupial and eutherian mammals diverged from each other about 160 Ma [1] (figure 1). One hypothesis is that the evolution and diversification of mammals has been driven by a series of chance events, retrotransposition integration and exaptation, to produce novel but essential placental genes [2]. This review addresses these questions on the origin and evolution of genomic imprinting in mammals, and its relevance to viviparity, placentation, lactation and post natal care.

2. Evolution of viviparity and the placenta

The placenta is defined as an organ of physiological exchange between the mother and fetus [3]. The placenta is probably the most varied structure in the

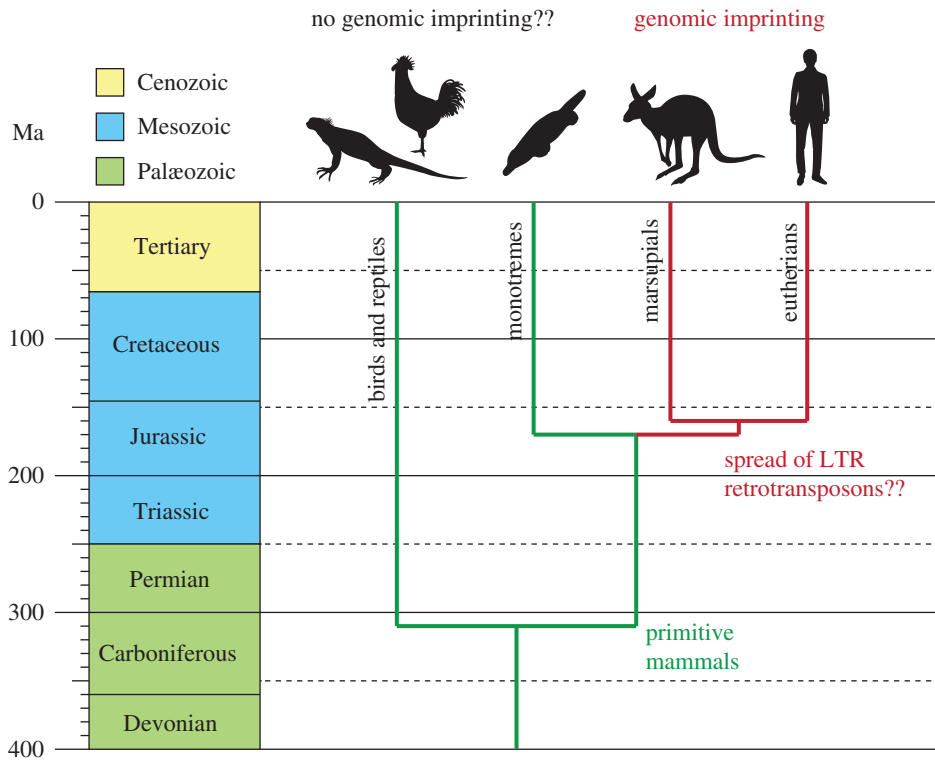


Figure 1. The timing of genomic imprinting acquisition and of the divergence of birds and reptiles, monotremes, marsupials and eutherians. The vertical axes represent the time line from 400 Ma to the present. The coloured boxes represent each geological period. The green and red lines represent the evolution of the groups with and without genomic imprinting. The silhouettes represent one example species from each group. LTR, long terminal repeat.

animal kingdom, and is found in a wide variety of taxa, even among invertebrates such as scorpions and lower vertebrates such as selachian sharks [4,5]. Placentation allows the production of live young, but it is often not recognized that this is not a uniquely mammalian characteristic. In amniotes, there are four fetal membranes: the amnion that surrounds the fetus and protects it from mechanical and physiological shock; the yolk sac that in birds and some reptiles surrounds the egg yolk; the allantois, which is an extension of the embryonic bladder and stores excretory products; and the chorion, which is formed by extraembryonic mesoderm and an outer trophoblast layer and fuses with either the yolk sac to form the chorio-vitelline placenta or with the allantois to form the chorio-allantoic placenta. Most mammals rely on both types of placenta at least for some periods of pregnancy, and even in humans the yolk sac is crucial for the survival of the early embryo [6]. It is the site of fetal blood and blood vessel formation, nutrient and gas exchange before the allantoic placenta is established, and transfer and biosynthesis of cholesterol and proteins [7–10]. Dysfunction or abnormal growth of the yolk sac can negatively influence success of the pregnancy or post natal health.

Although the majority of marsupials depend on a chorio-vitelline placenta, which is often considered somehow a less important placenta, it is fully functional and not only is responsible for the nutrients and gaseous exchange but also induces a maternal recognition of pregnancy [11,12] and elaborates many hormones [13,14]. The term placental mammal (preferably known as eutherian mammal) gives the incorrect impression that the placenta is found only in that mammalian infraclass. The invasiveness of the placenta varies from superficial to highly invasive but in all cases is critical for the survival of the fetus [13,15]. In general, allantoic placentae are more invasive of the uterine endometrium and non-invasive or less

invasive placentas were originally considered to be more primitive than highly invasive placentas [16]. This paradigm is now generally not accepted, since a common feature of the placenta of viviparous elasmobranchs, amphibians and reptiles is invasion and haemotrophic nutrition [4,15]. Indeed, in certain marsupials a greater reliance on histotrophic nutrition of a non-invasive placenta can be a derived (or more recently evolved) character [17]. A few marsupials, such as the bandicoot, dasyurids and wombats, have a chorio-allantoic placenta, which supplements the placental functions of the yolk sac or chorio-vitelline placenta [17–20]. In these species, the placenta is invasive to a variable degree, and in the bandicoot a maternal homokaryon is formed which later in development fuses with the trophoblast to form a fetomaternal syncytium at the site of the chorio-allantois [21].

Every mammalian species has a yolk sac that develops differently and has slightly different functions as a placenta. However, the nutritive, biosynthetic and hematopoietic functions of the human yolk sac are not new inventions specific to humans, but ancestral features shared with other viviparous mammals. The rodent visceral yolk sac (inverted yolk sac) is an important organ of maternal–embryonic exchange of amino acids, transferrin, vitamin B₁₂, calcium and other ions [22,23]. The rodent visceral yolk sac is also the route by which passive immunity (immunoglobulins) is passed to the embryo [24,25]. Agents that cause visceral yolk sac dysfunction during organogenesis can result in embryotoxicity and its damage leads to embryonic malformation [26]. In virtually all eutherian species studied, there is evidence of synthetic activity by the yolk sac and it serves as hematopoietic organ in all mammalian species studied (reviewed by King & Enders [22]). These synthetic activities may occur throughout gestation, influencing implantation as well as embryonic and fetal growth (reviewed by King & Enders [22]). In marsupials,

the yolk sac forms the definitive placenta, being responsible for physiological exchange, biosynthesis and endocrinology [17,20,27,28].

There are about 106 origins of viviparity within the squamates (lizards and snakes) [29,30]. Many of the viviparous reptiles have a highly invasive chorio-allantoic placenta, and the developing young depend on receiving nutrients via this placental attachment. Four independent origins of complex placentae have been identified in only one modern reptilian lineage, the lizard family Scincidae [31–34]. Some species have small eggs, complex, eutherian-like chorio-allantoic placentae and are highly placentotrophic [35]. There is significant uptake of nutrients across the placenta of species with complex chorio-allantoic placentae [36]. Indeed, in the viviparous skink of central Africa, placentation is endotheliochorial and is strikingly convergent on features of the placenta of viviparous mammals [37]. As yet, there is no evidence of genomic imprinting in reptiles, but the new data on reptile genes and genomics is awaited with interest [34,38].

3. Transfer of nutrients: a common theme in imprinting

(a) Placentation and pre natal imprinting

Most imprinted genes exhibit placental expression [39]. Several genetic experiments indicated that there are a number of imprinted genes that are essential for placental development and growth. Partial uniparental duplication of certain chromosome regions causes several developmental and growth abnormalities, probably owing to placental defects. Importantly, in mice, seven out of 21 imprinted regions are related to such phenotypes, such as early embryonic, mid-fetal or late fetal lethality and/or prenatal growth retardation/stimulation [40]. Subsequent knockout mice studies of many candidate imprinted genes confirmed that there are several essential placental genes in such imprinted regions. Among seven such imprinted regions, three imprinted regions are linked to embryonic lethality: proximal chromosome 6, distal chromosome 7 and distal chromosome 12. Embryonic lethality is observed in mice with maternal duplication of proximal chromosome 6 (MatDp(prox6)). *Peg10* (paternally expressed 10) is a major gene responsible for this early embryonic lethal phenotype. *Peg10* knockout (KO) mice show early embryonic lethality owing to severe placental abnormality with almost complete lack of labyrinth and spongiotrophoblast layers, the essential part of the placenta where nutrient and gas exchange occur between fetal and maternal blood cells [41]. *Peg10* encodes a protein with homology to the Gag and Pol proteins of certain long terminal repeat (LTR) retrotransposons [42]. Although its biochemical function is yet unknown, *Peg10* is an essential placenta-specific gene expressed paternally in mice. Paternal duplication of distal chromosome 7 (PatDp(dis7)) also causes early embryonic lethality. *Mash2/Ascl2* (mammalian *achaete scute* homologue 2/*achaete scute* complex like 2), one of the maternally expressed genes in this imprinted gene cluster, is responsible for the phenotype. There is severe placental abnormality in *Mash2* KO mice, such as a lack of labyrinth and spongiotrophoblast layers like *Peg10* KO mice in addition to an overgrowth of trophoblast giant cells, and they died

on day 10 of gestation [43]. *Mash2/Ascl2* encodes a basic helix–loop–helix transcription factor specifically expressed in the placenta. *Peg10* KO and *Mash2* KO mice have a similar placental phenotype, although *Mash2* is a maternally expressed gene while *Peg10* is a paternally expressed gene. It should be noted that *Peg10* and *Mash2/Ascl2* are the genes responsible for parthenogenetic and androgenetic death of mouse embryos, respectively [44–46]. Interestingly, mice with (MatDp(dis7)) have a disrupted phenotype and die at mid-fetal stages rather than the early embryonic lethality seen in (PatDp(dis7)). It is highly probable that both lack of paternally expressed *Igf2* (insulin-like growth factor 2) expression and overexpression of two maternally expressed genes, *Cdkn1c* (cyclin-dependent kinase inhibitor 1C, also known as *p57Kip2*) and *H19* are attributable to this phenotype. *Igf2* plays a role as one of major growth factors in fetal development. *Igf2* KO mice have severe growth retardation but the KO does not cause lethality alone [47]. *Cdkn1c* functions as a growth inhibitor. Both functional loss of *CDKN1C* and the overexpression of *IGF2* are attributable to Beckwith–Wiedemann syndrome, characterized by fetal overgrowth [48,49]. *Cdkn1c* also affects the growth of placental trophoblast giant cells [50]. *H19* is a well-conserved non-coding RNA (ncRNA) in both marsupials and eutherians [51], suggesting that it has some unknown important role in mammalian development although KO mice have no significant abnormal phenotypes [52]. *H19* may have tumour-suppressing activity when combined with overexpression of *Igf2* [53]. *H19* is highly expressed in both embryos and placentas, so its overexpression disturbs the gene expression profiles of many other genes that may affect on embryonic growth [54]. Phenotypes of both maternal and paternal duplication of distal chromosome 12 (MatDp(dis12)) are also related to the placental function. Mice with MatDp(dis12) have late embryonic lethality or neonatal lethality associated with growth retardation. *Peg11/Rtl1* (paternally expressed 11/retrotransposon-like 1) is a major gene responsible for these phenotypes. Half of *Peg11/Rtl1* KO mice have late fetal lethality and another half have neonatal lethality associated with late fetal growth retardation [55]. This is because of the fetal capillary abnormality in the labyrinth layer. Endothelial cells in the fetal capillaries are phagocytosed by surrounding trophoblast cells and clogged at many sites, indicating that *Peg11/Rtl1* is essential for the maintenance of the feto-maternal interface of the placenta during gestation. *Dkl1/Peg12* may also contribute to these phenotypes because in KO mice there is partial neonatal lethality associated with growth retardation [56,57]. Mice with PatDp(dis12) have late embryonic lethality associated with abnormal fetal morphology and placental enlargement [58,59]. In this case, the major cause is overexpression of *Peg11/Rtl1* due to loss of maternally expressed *antiPeg11/antiRtl1*. *AntiPeg11/antiRtl1* is ncRNA but involves at least six micro RNAs targeting to *Peg11/Rtl1* by RNA interference. Thus, loss of *antiPeg11/antiRtl1* leads to 2 to 4 fold accumulation of *Peg11/Rtl1* mRNA. *AntiPeg11/antiRtl1* KO mice exhibited neonatal lethality and placental overgrowth [55]. In the labyrinth layer of *antiPeg11/antiRtl1* KO mice, expansion of the fetal capillary size associated with severely damaged surrounding trophoblast cell layers was observed. In the case of paternal duplication (or paternal disomy), double dosage of *Peg11/Rtl1* without maternally expressed *antiPeg11/antiRtl1* leads to 4–6 fold increment of *Peg11/Rtl1* mRNA, causing

Table 1. List of genes that are imprinted in eutherian mammals tested for imprinting in marsupial mammals.

gene	eutherian reference	marsupial reference
imprinted in marsupials		
<i>with a DMR</i>		
<i>H19</i>	Zhang & Tycko [72]	Smits <i>et al.</i> [51]
<i>PEG10</i>	Ono <i>et al.</i> [42]	Suzuki <i>et al.</i> [73]
<i>without a DMR</i>		
<i>IGF2</i>	DeChiara <i>et al.</i> [47]	O'Neill <i>et al.</i> [74]
<i>IGF2R</i>	Barlow <i>et al.</i> [75]	Killian <i>et al.</i> [76]
<i>INS</i>	Moore <i>et al.</i> [77]	Ager <i>et al.</i> [78]
<i>PEG1/MEST</i>	Kaneko-Ishino <i>et al.</i> [79]	Suzuki <i>et al.</i> [80]
not imprinted in marsupials		
<i>PHLDA2 (IPL)</i>	Qian <i>et al.</i> [81]	Suzuki <i>et al.</i> [82]
<i>PPP1R9A (Ppp1r9a)</i>	Nakabayashi <i>et al.</i> [83]	Suzuki <i>et al.</i> [73]
<i>SGCE</i>	Müller <i>et al.</i> [84]	Suzuki <i>et al.</i> [73]
<i>SNRPN</i>	Leff <i>et al.</i> [85]	Rapkins <i>et al.</i> [86]
<i>UBE3A</i>	Herzing <i>et al.</i> [87]	Rapkins <i>et al.</i> [86]
<i>DLK</i>	Wyllie <i>et al.</i> [88], da Rocha <i>et al.</i> [89]	Weidman <i>et al.</i> [90], Edwards <i>et al.</i> [91]
<i>DIO3</i>	Tsai <i>et al.</i> [92], da Rocha <i>et al.</i> [89]	Edwards <i>et al.</i> [91]
not expressed in marsupials		
<i>RTL1 (Rtl1;PEG11)</i>	Seitz <i>et al.</i> [93]	Edwards <i>et al.</i> [91]
no orthologue found in marsupials		
<i>Air/Airm</i>	Lyle <i>et al.</i> [94]	Weidman <i>et al.</i> [95]
<i>MEG3</i>	Miyoshi <i>et al.</i> [96]	Weidman <i>et al.</i> [90]
<i>NNAT</i>	Kagitani <i>et al.</i> [97]	Evans <i>et al.</i> [98]
<i>PEG3</i>	Kaneko-Ishino <i>et al.</i> [79]	Suzuki <i>et al.</i> [99]

more severe abnormal phenotypes. Thus, both the lack and overexpression of *Peg11/Rtl11* are also responsible for the various phenotypes observed in MatDp(dis12) and PatDp(dis12), respectively. The same is true for human patients with MatDp and PatDp of chromosome 14, orthologous to mouse chromosome 12 [57].

Several imprinted genes so far described are important in the transfer of nutrients across the placenta. Genes that increase growth are usually paternally expressed, such as *Igf2*, *Peg1/Mest*, *Peg10*, *Peg3/Pw1*, *Kcnqlot1/Lit1*, *Rasgrf1*, *Zac1*, *Peg11/Rtl1*, *Dlk1*, while genes that tend to restrict growth are maternally expressed, such as *Phlda2*, *Igf2r*, *Meg1/Grb10*. This was the basis of the original parental conflict theory, which proposed that the paternally inherited genome would be modified to increase the growth of his offspring to increase his genetic fitness [60]. Conversely, the maternal genome would be modified to restrict resources to any one young or litter, allowing her to carry many successive pregnancies, thus increasing her genetic contribution to the next generation.

Another theory, the co-adaptation hypothesis, explains how paternally expressed imprinted genes have been maintained at some loci due to co-adaptation of the maternal hypothalamus and the placenta [61–63]. This is supported by the observations that *Peg3* (paternally expressed gene 3), which is involved in maternal care, placental nurturing and regulating milk let-down in the female, also functions in the hypothalamus of the neonate to regulate attachment to the nipple and sucking

behaviour [61–66]. The hypothalamus may be a more important site for genomic imprinting than previously recognized, and involved in long-term programming of hypothalamic functions [67]. Further studies are awaited with interest.

Recently, the paternal transmission of X-linked *RLIM/Rnf12* gene encoding a nuclear ubiquitin ligase is reported to be essential for mammary gland development because the paternal X chromosome is selectively activated in the mammary gland [68]. Usually, in eutherian mammals random X chromosome inactivation occurs in female tissues and organs while paternal X chromosome is selectively inactivated in the placenta. In contrast, in marsupials, X-inactivation is paternal [69]. Interestingly, certain regions in female brain have a bias to silence the paternal X chromosome [70,71]. The biological meaning of this phenomenon is unclear at the moment and will be addressed when such an organ-specific paternal X chromosome activation/inactivation does occur in other reproductive organs and tissues, such as uterus, ovary and specific sites of the brain. As *PEG3* is an autosomal imprinted gene showing constant paternal expression in all the tissue and organs, the situation may be different in this case.

In marsupials, about 18 genes that are imprinted in eutherians have so far been tested (table 1), not including X-inactivation. Of these 18, only six have genomic imprinting: *H19* and *PEG10* (see below for more details) have differentially methylated regions (DMRs) and *IGF2*, *IGF2R*, *PEG1* and *INS* do not [51,73,74,76,78,80,100]. All of these are expressed in

the marsupial placenta except for *IGF2R* which as yet has not been examined in the placenta. Insulin (*INS*) and insulin-like growth factor regulate growth of the placenta and the young. Tamar wallaby *INS*, like *INS* in humans and *Ins2*, is expressed in the yolk sac placenta [78]. This was thought to be the exclusive site in which this gene is imprinted [77]. However, we have demonstrated imprinting in other tissues (see the next section).

A number of other genes imprinted in eutherians are not imprinted in the marsupial placenta (table 1). *PHILDA2*, for example, is another gene, like *Rhl1*, which is essential for placental formation and function, yet neither gene is imprinted in marsupials and *Rhl1* is not expressed [55,82,91]. The *DLK1-DIO3* imprinting cluster appears to have arisen with the introduction of a new gene only in the eutherian cluster [91]. The marsupial locus is twice the size of the eutherian one, because of the accumulation of LINE repeats. *SNRPN* in the tamar and *UBE3A* in the tamar and in the platypus are not imprinted [86], and the rearrangement that brought *UBE3A* and *SNRPN* together must have occurred in the eutherian ancestor after the two groups of therian mammals diverged from each other.

No orthologue has been found for the ncRNA *Air/Airn*, or for the genes *NNAT* or *MEG3* [90,95,98]. *PEG3* cannot be identified in the tamar genome possibly because of poor genomic coverage of this region [99,101]. These differences in the extent of imprinting may reflect the different reproductive strategies of these two infraclasses of therian mammals, since marsupials have a uniformly short gestation. However, they have an extremely sophisticated and lengthy lactation, during which milk composition changes dramatically [20], and the maternal control of post natal growth is absolute [102,103]. Perhaps there are as yet undiscovered imprinted genes in marsupials that are not imprinted in eutherian mammals.

(b) Post natal imprinting and behaviour

Viviparity results in the birth of live young. In mammals, the maternal support of the young extends beyond birth into a period in which there is extensive post natal care of the young, supported by the secretion of milk. Nutrient transfer via a modification of the maternal physiology may be the key mammalian characteristic, using both placenta and mammary gland to do so. However, in the pigeon there is a crop milk produced from the oesophagus that is rich in lipids and protein and which sustains the young squab. Similarly, flamingos and especially penguins also produce crop milk. However, these secretions are produced by both sexes, and so are not in any way analogous to mammalian milk. Thus, in mammals it is the convergence of viviparity, placentation and lactation in the female that has led to their successful mode of reproduction.

The mammary gland is thought to have evolved initially as a small integumental gland that synthesized antimicrobial secretions, and that the nutritional role evolved subsequent to its protective function [104]. It now seems probable that it evolved from the innate immune system [105]. The mammary gland regulates post natal nutritional transfer by a positive feedback loop with the mother's brain in response to the sucking stimulus, in a similar way to that observed between the placenta, fetus and the maternal hypothalamus. There are monoallelically expressed imprinted genes in the mammary gland that are mis-regulated in breast cancer [106,107]. We have recently shown that two genes intimately

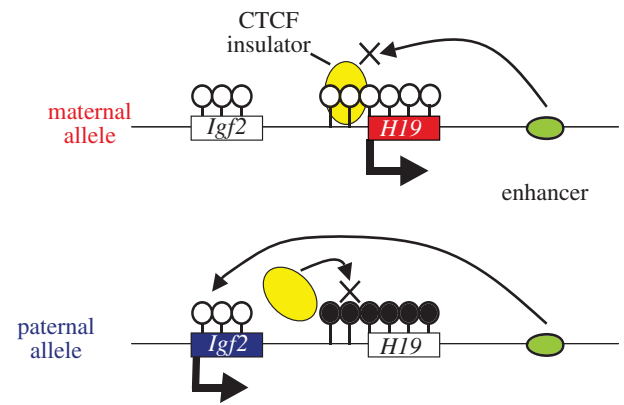


Figure 2. Regulation of the paternal and maternal expression in the *Igf2-H19* imprinted domain by a single gDMR. The open lollipops represent unmethylated CpG islands and the black lollipops represent methylated CpG islands. The boxes represent *Igf2* and *H19* genes and the arrows from the boxes indicate expression of the genes. The yellow and green circles represent the CTCF insulator protein and a downstream enhancer, respectively.

involved in growth and metabolism, *INS* and *IGF2*, are both tissue-specifically imprinted in the marsupial mammary gland and liver [108]. *GRB10* binds to the *INS* and *IGF1* receptors and inhibits the growth-promoting activities of *INS* and *IGF1* and 2 [109]. Disruption of *GRB10* in mice causes fetal and placental overgrowth [110–112]. Thus, *GRB10* may also regulate mammary gland growth and ultimately milk production through its inhibition of *INS* and *IGF2*. Genomic imprinting in the mammary gland therefore may be as critical for regulating post natal growth as it is for regulating pre natal growth in the placenta. These data also support the co-adaptation hypothesis. It is tempting to speculate that the acquisition of imprinting in the mammary gland might have contributed to the development and elaboration of lactation as a key reproductive strategy amongst mammals.

4. Evolution of mammalian imprinting

(a) Germline DMR, a master key for imprinting control

Imprinted genes are most often seen in clusters termed imprinted regions or domains. Imprinted expression of multiple genes in an imprinted domain is coordinately regulated by a single genomic element called the gDMR or ICR (imprinting control region), even in the case that an imprinted region consists of both paternally and maternally expressed genes (*PEGs* and *MEGs*). gDMRs are CpG-rich and differential DNA methylation is observed between two parental alleles. The difference in DNA methylation on gDMRs is established during gametogenesis and is maintained throughout development. There are two types of gDMRs, one is paternally methylated and the other is maternally methylated. Eighteen loci are associated with maternal methylation and all these maternal gDMRs are located at promoters while only three loci have paternally methylated gDMRs and are located in intergenic regions (The *Gpr1-Zdbf2* locus, which was previously thought to be the fourth paternally imprinted region, has recently been reported as a maternally imprinted region) [113,114]. In the *Igf2-H19* domain, the paternally methylated gDMR represses *H19* expression from the paternal allele, but on the other hand it induces upstream *Igf2* expression from the paternal allele inhibiting the binding of an insulator protein, CTCF, which

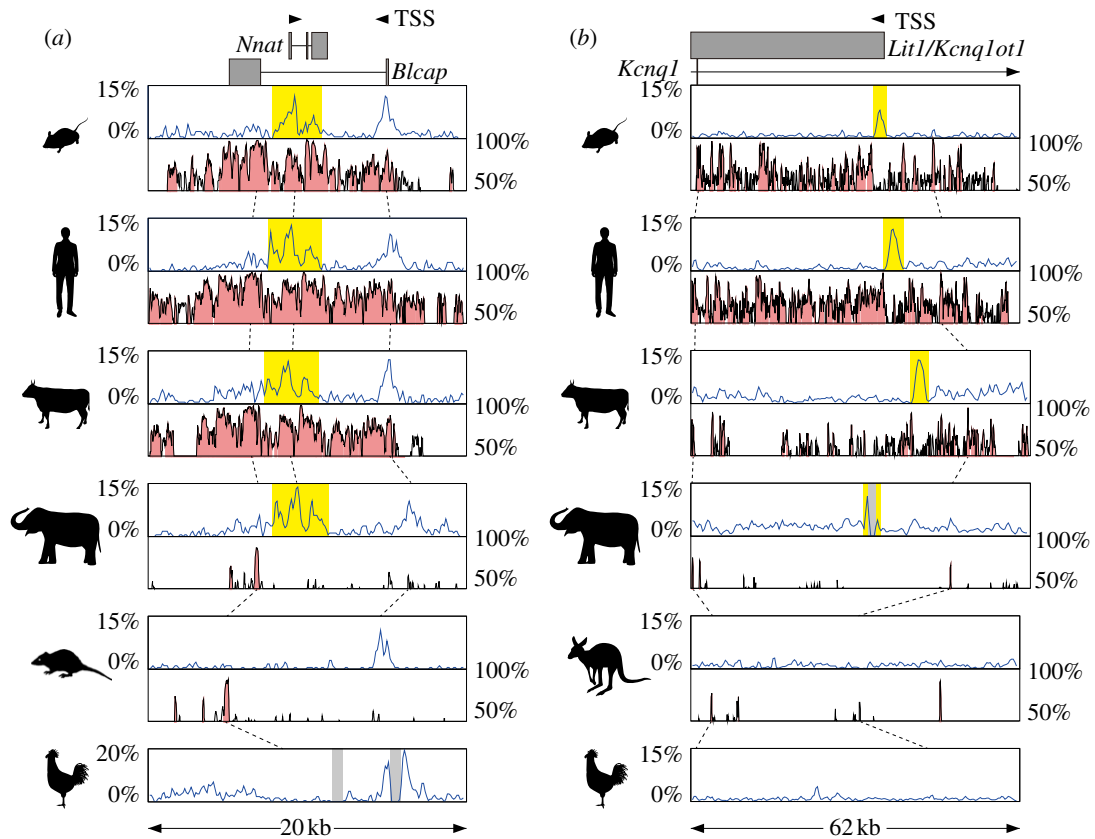


Figure 3. (a) Comparison of CpG contents and conservation among the orthologous genomic regions around the *Nnat* gDMR. The upper blue graphs show the CpG content in the genomic sequences. The lower pink graphs show conserved regions in the genomic sequences between one species and the other species located just below (eg. the pink graph seen in the mouse row is the comparison of mouse (base) and human and the graph in the human row is the comparison of human (base) and cow). The broken lines indicate where some conservation peaks in the upper row correspond in the next lower row. The arrowhead indicates the transcription start site (TSS) with the direction and the grey box shows the exon. Gaps in the sequences are represented by the light grey shadows in graph regions. The CGI forming gDMR in the mouse and the corresponding CGI in other species are highlighted in yellow. (b) Comparison of CpG contents and conservation among the orthologous genomic regions around the *Lit1/Kcnq1ot1* gDMR. Explanations for the each component are the same as (a).

blocks the action of downstream enhancer (figure 2). Thus, DNA methylation of a single gDMR can induce the *Igf2* (*Peg*) expression and the *H19* (*Meg*) repression at the same time in this regulatory mechanism known as the insulator model (figure 2). Large ncRNA-mediated epigenetic silencing is another important regulatory mechanism to control domain-wide imprinted expression by a single gDMR. In the *Kcnq1* and *Igf2r* domains, the maternally methylated gDMRs induce paternal expression of the large ncRNAs named *Lit1/Kcnq1ot1* and *Airn/Air*, respectively, and these ncRNAs mediate domain-wide silencing only on the paternal allele by recruiting repressive epigenetic modifiers such as G9a [115,116]. Because differential methylation of gDMRs plays the most critical role in the imprinting regulatory mechanism, the acquisition of gDMRs in the genome must be a pivotal event for the evolution of imprinting in mammals. The emergence of imprinted regions must have been related to the emergence of gDMR in the mammalian genome. It is therefore quite important to elucidate how the gDMRs emerged in the imprinted region and what the origin of gDMR sequences is.

(b) Biological importance of the regulatory mechanisms

Both *Pegs* and *Megs* are often contained in a single imprinted domain as described above. Regardless of the mechanisms directly regulating imprinted expression of each gene, it is true that only *Pegs* or *Megs* are expressed from the chromosome with a methylated or unmethylated gDMR. Therefore, the only way that the complete set of *Pegs* and *Megs* can be

expressed in a single diploid cell is that each parental chromosome has a reciprocal methylation pattern of gDMRs. The imprinted regions are unique regions where epigenetic complementation by two parental chromosomes is necessary. As many imprinted genes are essential for mammalian development, this is the reason why mammals cannot lose genomic imprinting once the complex gene expression pattern has evolved at imprinted regions [117].

(c) Retrotransposons, novel CpG islands and gDMRs

In therian genomes, almost half of the genomic sequences contain traces of transposable elements such as transposons and retrotransposons [118]. It has long been suggested that the molecular mechanisms underlying genomic imprinting evolved from host defence epigenetic mechanisms including small RNAs, large ncRNAs, DNA methylation and chromatin modification against these transposable elements [119–122]. Genomic imprinting is found in some plants (eg. *Arabidopsis* sp., *Zea mays*), some insects and in some mammals. However, it is most widespread in mammals, and almost all of the imprinted genes described are expressed in the placenta. Interestingly, a failure of the genome defence mechanism to regulate the expression of retroelements can lead to placental defects, as recently demonstrated in hybrids between *Mus musculus* and *Mus caroli*, in which the DNA methylation is lost [123]. In both plants and animals there appears to be a correlation of imprinting with the ratio of transposable elements. Once imprinting is established, imprinted domains can develop in

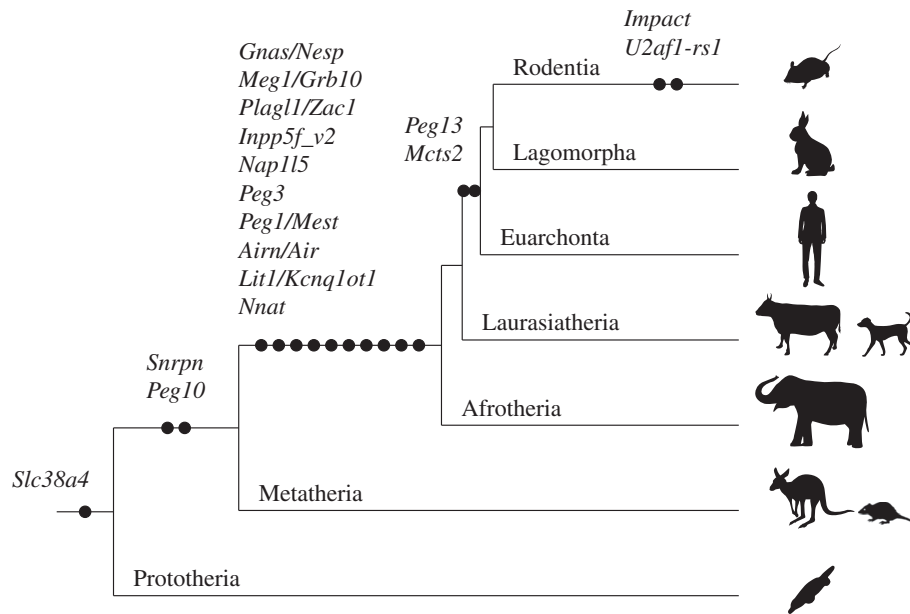


Figure 4. The timing of the novel CpG island emergence in each maternal gDMR locus during mammalian evolution. The black circles represent the acquisition of novel CGI to the locus. The genes associated with novel CGI emergence are shown above the black circles. The silhouettes represent several example species from each group.

several ways [124]. Imprinting can spread to adjacent regions in a stepwise manner, or can develop by genome reorganization. Divergent evolution of the domain can also influence the acquisition of imprinting (reviewed in [125]). In monotremes, the arrangements of the gene clusters that are imprinted in eutherians are conserved in other vertebrates [126,127], but the distribution of repeats including LTR and DNA elements has significantly expanded in only therian mammals [126]. There are several reports suggesting that retrotransposition is involved in the acquisition of a gDMR. We previously reported that the insertion of *Peg10*, a retrotransposon-derived imprinted gene essential for placental development in the mouse, must have occurred in therian ancestors after the divergence of marsupials and eutherian mammals from monotremes [73]. The CpG island (CGI) forming the *PEG10* DMR has also newly emerged in the therian ancestor [73]. The *IGF2-H19* imprinted region is also conserved in both marsupials and eutherians and *H19* genes as well as *H19* DMRs emerged in the therian ancestor [51]. These genomic data demonstrated that *PEG10* and *IGF2-H19* imprinted regions are the oldest among all the imprinted regions during therian evolution and both the maternal gDMR (*PEG10* DMR) and paternal gDMR (*H19* DMR) were established when the genomic imprinting started at least 160 Ma. Importantly, they are essential for embryonic and placental growth and development, as mentioned above. Also some of the small imprinted genes that reside in an intron of other genes, such as *Mcts2*, *Nap115*, *Inpp5f_v2*, *U2af1-rs1* and *Nnat*, are thought to be inserted into their present positions by retrotransposition [98,128] (figure 3). Interestingly, in every case, the CGIs forming the gDMR probably emerged as novel CGIs at the same time as the retrotransposition of each gene occurred. Therefore, we recently examined the generality of the hypothesis that the CGIs forming gDMRs were newly acquired during mammalian evolution by reviewing the time of novel CGI emergence for all the maternal gDMR loci using new and published data [99]. The comparative sequence analyses suggested that emergence of novel CGIs occurred universally in the maternal gDMR loci at different time points during mammalian evolution (figures 3 and 4). In most loci,

the novel CGIs emerged in introns but in *Slc38a4*, *Snrpn* and *Gnas* loci CGIs were unlikely to have emerged in introns in the ancestral mammal and differential methylation was acquired only in the eutherian lineage. In the eutherian lineage, interestingly, the location of the CGIs became internalized within the transcription unit by the acquisition of the IC transcript and *Nesp* to *Snrpn* and *Gnas* loci, respectively. This is consistent with the recent studies showed that transcription is required to establish maternal imprinting at these loci [129,130]. Thus, the emergence of a novel CGI in an intron of an existing gene or the acquisition of upstream transcript over an existing CGI might be part of the evolutionary pathway for emergence of gDMRs. Not all loci have evidence of involvement of retrotransposition events for the acquisition of gDMRs, but considering the number of gDMRs associated with retrogenes, retrotransposition appears to be a key mechanism by which novel CGIs have been acquired. However, the mechanistic link between retrotransposition, novel CGI acquisition and differential methylation is largely unknown. The mechanism of novel CGI emergence may be more complex than the suggestion that CpG sequences originated solely by insertion of GC-rich retrotransposons [99]. Further investigations will be required to clarify how these phenomena can be connected.

5. Conclusions

Genomic imprinting in mammals is essentially a conserved process, but has greatly been expanded in the Eutheria. It is clear that imprinting arose at many different time points in mammalian evolution due to different selective pressures at different loci and that it is continuing to evolve. It is also clear that there is a great diversity of roles that imprinted genes play. Acquisition of novel CGIs appears to be a key genomic change for the evolution of genomic imprinting that generally occurred in the maternal gDMR loci. Novel CGIs may also have emerged in genomic loci other than in imprinted domains, but only became differentially methylated under certain conditions, contributing to

the diversification of mammals. This might explain why imprinted genes are often associated with fetal–maternal nutrient transfer, placental development, viviparity, lactation

and the mammalian-specific maternal behaviour associated with suckling young that must have evolved for mammals to survive.

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