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The Regulation and Biological Significance of Genomic Imprinting in Mammals

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Genomic imprinting is a system of non-Mendelian inheritance that is unique to mammals. Two types of imprinted genes show parent-of-origin-specific expression patterns: the paternally expressed genes (*Pegs*), and the maternally expressed genes (*Megs*). Parental genomic imprinting memory is maintained in the somatic cell lineage and regulates the expression of *Pegs* and *Megs*, while it is erased and re-established in the germ cell lineage according to the sex of the individual. The paternal and maternal imprinting mechanisms, which regulate different sets of *Pegs* and *Megs*, are essential for establishing the parental expression profiles of imprinted genes that are observed in sperms and eggs. Based on recent evidence, we outline the relationship between parental imprinting and the expression profiles of *Pegs* and *Megs* and discuss a novel view of the regulation of genomic imprinting. We also discuss the biological significance of genomic imprinting and propose hypotheses on the essential nature of genomic imprinting and the close relationship between genomic imprinting and the acquisition of placental tissues during mammalian evolution.

Key words: complementation hypothesis, evolution, development, genomic imprinting, parental imprinting, *Peg* and *Meg*, placenta hypothesis.

The concept of genomic imprinting, which relates to the functional differences between paternal and maternal genomes in mammals, was proposed in 1984 by Surani *et al*. (*1*) and Solter *et al*. (*2*). These two groups demonstrated that gynogenetic (parthenogenetic) or androgenetic embryos that had either two maternal or paternal pronuclei showed early embryonic lethality and never developed to term. Cattanach *et al*. (*3*) provided strong genetic evidence for this idea, by showing that mice with uniparental duplication of specific chromosomal regions displayed a variety of defects in development, growth, and behavior. Thus, more than ten chromosomal imprinted regions in the mouse genome (*4*) and the corresponding syntenic regions in the human genome (*5*) have been identified. In 1991, three functionally or positionally related genes, *Igf2*, *Igf2r*, and *H19*, were identified as imprinted genes whose expression was parent-of-origin-specific (*6*, *7*, *8*). Several human genetic diseases that entail non-Mendelian inheritance, such as Prader-Willi syndrome (PWS), Angelman syndrome (AS), and Beckwith-Wiedemann syndrome (BWS), have been linked with uniparental chromosomal duplications (*9*, *10*), and several imprinted genes have been identified in these regions. Systematic screening methods for imprinted genes have contributed to the identification of novel imprinted genes and to the precise localization of imprinted regions (*11–18*). The fact that most imprinted genes have been located within established imprinted chromosomal regions (*4*) strongly supports the idea that genomic imprinting is due to the existence of imprinted genes that show uniparental expression patterns. In this review, we summarize recent studies on the regulation of genomic imprinting, and we provide a novel perspective on the relationship between the expression profiles of *Pegs* and *Megs* in somatic cell lineages and the paternal and maternal imprinting mechanisms in germ cell lines. Given the accumulated knowledge of imprinted genes and their regulatory networks, reconsideration of the biological significance of genomic imprinting is timely and appropriate.

1. Genomic imprinting: the relationship between parental imprinting and parent-of-origin-specific expression profiles

1.1. Two different imprinted gene classifications— Imprinted genes are defined as genes that are expressed in a parent-of-origin-specific manner. Parthenogenetic or androgenetic embryos are excellent sources of imprinted genes, because paternally expressed genes are not expressed in the former, and maternally expressed genes are not expressed in the latter. Imprinted genes have been isolated systematically using various methods (*11–18*). We used subtraction-hybridization to categorize the genes into two groups: *Pegs* (paternally expressed genes), and *Megs* (maternally expressed genes) (*15–18*). This nomenclature is very simple and avoids any conceptual confusion between 'imprinted' and 'repressed'. In this review, we use the examples of *Pegs* and *Megs* to explain the relationship between parental imprinting and the expression profiles of imprinted genes.

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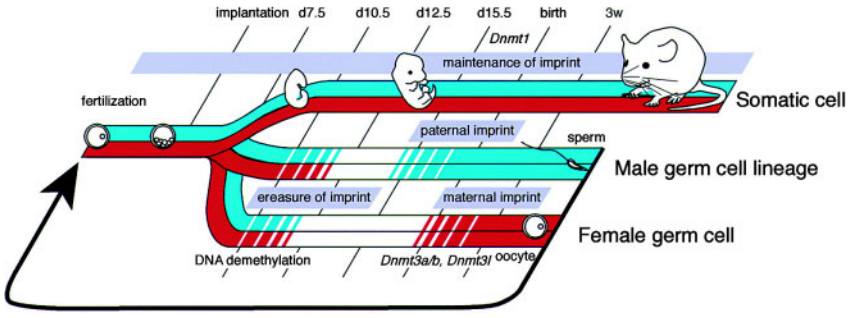

Fig. 1. **Genomic imprinting during the mammalian life cycle is associated with DNA methylation.** Genomic imprinting memory is stably maintained in somatic cell lineages, whereas it is erased and re-established in germ cell lines according to the sex of the individual. The erasure process occurs in day-10.5 to -12.5 PGCs in both male and female germ lines (*27*). The maternal imprints in oocytes are established during oocyte maturation (*20*, *21*). As far as DNA methylation status is concerned, the paternal imprint on the *H19* region appears to be established before birth (*85*, *86*, and Lee, unpublished data). DNA demethylation, *de novo* methylation (paternal and maternal imprints), and maintenance methylation in somatic cell lineages are illustrated. However, other DNA methylation processes that are associated with mammalian development, such as DNA demethylation after fertilization and *de novo* methylation after implantation, are not shown.

Previously, the terms "maternally imprinted gene" and "paternally expressed gene" were used interchangeably, as were the terms "paternally imprinted gene" and "maternally expressed gene. " However, it is now apparent that the designations "maternally (or paternally) imprinted gene" and "paternally (or maternally) expressed gene" represent distinct classes of imprinted genes (see Table 1). The former is based on regulatory systems for parental imprinting in germ cells, and the latter is based on the expression profiles of imprinted genes in somatic cells (Fig. 1). Importantly, the term 'imprinted gene' when used with respect to germ cell lines does not necessarily mean that the gene is repressed in somatic cells (as described below). We adopt this dual classification scheme to elucidate the overall system of genomic imprinting. We also discuss the role of DNA methylation during the establishment and erasing processes of genomic imprinting memory in germ cells, and its maintenance in somatic cells (Fig. 1).

1.2. Establishment of maternally imprinted memory during oocyte maturation—Parthenogenetic (or gynogenetic) embryos that contain two nuclei from matured oocytes can develop up to day 9.5 but have very poor placental development, probably due to the total lack of *Peg* gene expression (*1*, *2*, *15*). Interestingly, Kono *et al*. (*19*) produced reconstituted embryos that contained nuclei from non-growing (ng) oocytes and from full-grown (fg) oocytes, and demonstrated that these embryos developed up to day 12.5 and had placentas of normal appearance. Our systematic analysis of both *Pegs* and *Megs* in these embryos revealed that most of the *Peg* genes were expressed at levels that were almost identical to those of normal fertilized embryos (*20*). Importantly, *Peg* expression in these embryos was derived from the nuclei of ng oocytes rather than fg oocytes. On the other hand, most of the *Megs* were expressed exclusively from the fg oocytes. Therefore, it is clear that the imprinted genes in ng oocytes are regulated differently from those in fg oocytes, in which maternal-type imprinting is already established, thus indicating that maternal memory is established during oocyte maturation (*20*).

These results demonstrate clearly that both *Pegs* and *Megs* are controlled during oocyte maturation by a maternal imprinting mechanism that represses *Pegs* and activates *Megs*. Recently, the repression and activation processes that take place during oocyte maturation were clearly demonstrated using reconstituted parthenogenetic embryos that consisted of nuclei from different stages of maturating oocytes and from an fg oocyte (*21*).

1.3. Default state for genomic imprinting in day-12.5 to -13.5 primordial germ cells—Parental memory must be erased during germ cell development so that the new genomic imprint can be established. There is compelling evidence that the erasure of genomic imprinting memory occurs in the primordial germ cells (PGCs) of developing embryos. Analyses of the differentially methylated regions (DMRs) of imprinted genes (*22*), and of imprinted gene expression in PGCs (*23*), suggest that erasure occurs in day-8.0 to -12.5 PGCs. Analysis of imprinted gene expression in embryonic germ (EG) cells that were established from several stages of PGCs (*24*, *25*), and nuclear transfer experiments using male PGCs (*26*), cor-

Table 1. **Classification of imprinted genes.** Based on their expression profiles in the default state of genomic imprinting in day-12.5 PGC clones, the *Pegs* and *Megs* were classified into two groups: the maternally imprinted and paternally imprinted genes. In embryos that have the default state of genomic imprinting (day-12.5–13.5 PGC clones), the *Pegs* under maternal imprinting and *Megs* under paternal imprinting show biallelic expression, and are thereby repressed by the maternal and paternal imprints, respectively. In contrast, the *Megs* under maternal imprinting and *Pegs* under paternal imprinting are silenced, and are activated by the maternal and paternal imprints, respectively. It should be noted that parental imprinting is indispensable for the expression of the latter group of imprinted genes.

	Peg (Paternally expressed genes)	*Meg* (Maternally expressed genes)
Maternally imprinted genes (during oocyte maturation)	*Peg1/Mest, Peg3, Peg5/Nnat, Peg10, Snrpn*, Ndn*, Zfp127*, Impact*, Kcnq1ot1**	*Meg1/Grb10, p57Kip2, Igf2r, Mash2, Ipl*, NESP55**
Paternally imprinted genes (during spermatogenesis)	*Igf2, Peg9/Dlk1, Rasgrf1**	*H19, Meg3/Gtl2*

*Additional data from the mouse ng/fg reconstituted embryos, *Dnmt3L* mat-KO embryos, and human biCHMs.

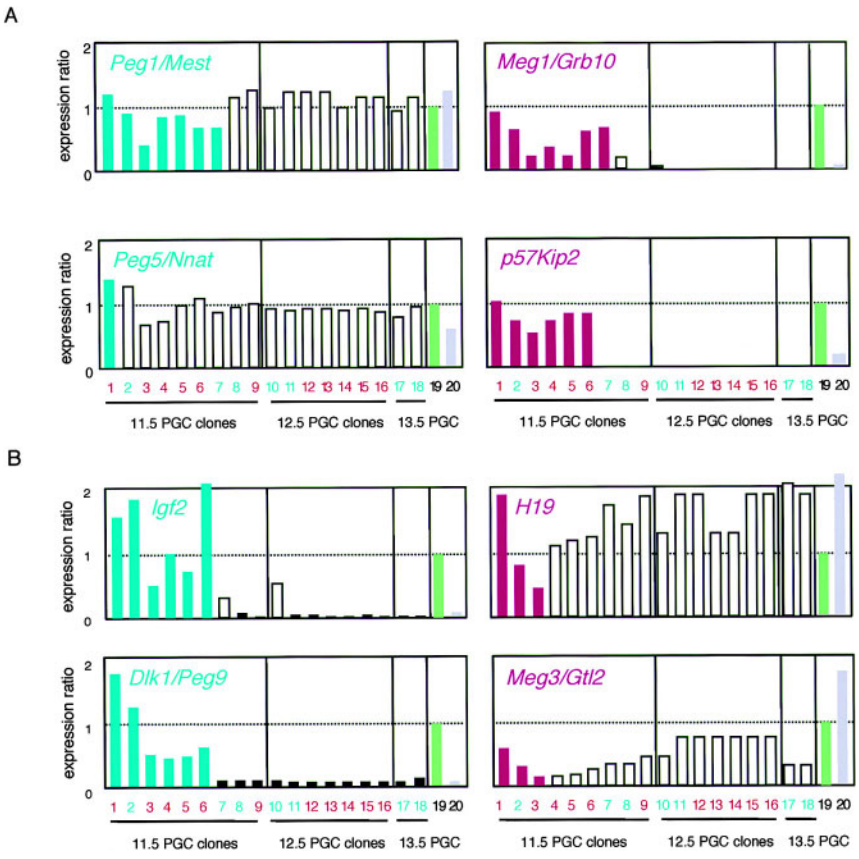

Fig. 2. **The erasure process and default state for genomic imprinting.** The erasure of genomic imprinting memory is represented in a series of day-11.5 PGC clones, and follows the default state in day-12.5 to -13.5 PGC clones (*27*). The blue and red bars indicate paternal and maternal gene expression, respectively, and the white and black bars indicate biallelic gene expression and non-expression (or insignificant levels of expression), respectively. The expression levels in *Dnmt1* KO embryos (gray bars) and those in the wild-type embryos (green bars) are shown in the two lanes furthest to the right. Imprinted genes that are under the control of maternal imprinting (A) and paternal imprinting (B) are shown (see Table 1). The expression ratio of 1 indicates the expression level of monoallelic expression in normal day 9.5 embryos.

roborate the hypothesis of genomic imprinting erasure in PGCs. The expression profiles of imprinted genes in EG cells and PGC embryos are almost identical to those in ng oocytes. However, the precise timing of the erasure process, and the exact nature of the default state of genomic imprinting are not yet fully understood.

Recently, we demonstrated a default state for genomic imprinting in mouse embryos that were produced by somatic cloning from day-12.5 to -13.5 PGCs (*27*). The day-12.5 and -13.5 cloned embryos showed early embryonic lethality (around day 9.5), and all of the imprinted genes lost their parent-of-origin-specific monoallelic expression patterns, i.e., either the biallelic or non-expression pattern (Fig. 2). The cloned embryos from both male and female PGCs showed the same developmental abilities and identical expression profiles for imprinted genes. Our results reflect those of Kato *et al*. (*26*), although they did not clearly show biallelic expression in day-14.5 to -16.5 male PGC embryos.

Pegs and *Megs* can be classified into two groups according to their expression profiles in the default state (Fig. 2 and Table 1). The first group contains *Pegs* that are expressed biallelically and *Megs* that are silenced, and the second group contains *Pegs* that are silenced and *Megs* that are expressed biallelically. Most *Pegs* and *Megs* belong to the first group. Since their expression profiles are identical to those derived from sperms, they should change to the maternal type during oocyte maturation when inherited maternally. On the other hand, the expression patterns of certain genes, such as *H19*, *Igf2*, *Meg3/Gtl2*, and *Peg9/Dlk1*, are identical to those of matured oocytes. Thus, when inherited paternally, the gene expression patterns of these genes should change to the paternal type during spermatogenesis. Therefore, it is reasonable to classify the former as a group of maternally imprinted genes and the latter as a group of paternally imprinted genes. This classification of imprinted genes is essentially the same as that derived using ng/fg reconstituted embryos (described above), because we have previously shown that *H19* and *Igf2* are the exceptions to the maternal imprinting rule (*20*). Thus, we conclude that during the mammalian reproduction cycle, both paternal and maternal imprinting memories are erased in PGCs and later re-established during gametogenesis *via* two independent mechanisms (paternal imprinting and maternal imprinting), thereby producing the parental expression profiles that are characteristic of male- and female-derived alleles (Fig. 1). It should be noted that the default state of genomic imprinting does not mean that all imprinted genes are expressed. About half of the imprinted genes are in the silent state when parental memory is erased (Table 1), and they require either maternal or paternal imprinting during gametogenesis for expression in subsequent generations. This finding indicates that both paternal and maternal imprinting are essential mechanisms for mammalian development and growth. We discuss the significance of genomic imprinting from this point of view in a later section (see Section 2.5).

1.4. Erasure of the genomic imprinting memory—The process of genomic imprinting memory erasure was first demonstrated in day-11.5 PGC clones (Fig. 2) (*27*). Com-

pared with the poor developmental abilities of the day-12.5 to -13.5 PGC clones, we found that some of the day-11.5 PGC clones developed without any morphological abnormalities to at least day 12.5, although no living pups were obtained. Interestingly, individual day-11.5 PGC clones showed different expression profiles for imprinted genes. Some of the clones showed expression profiles that resembled those of normal somatic cells, some showed expression profiles that were very similar to the default state observed in day-12.5 to -13.5 PGC clones, and others showed intermediate profiles. This indicated that the erasure of genomic imprinting memory proceeds in day-11.5 PGCs, and that the various stages of the process are represented in these series of PGC clones (Fig. 2).

This notion was supported by DNA methylation analyses of the PGC clones and the PGCs themselves. Analysis of three DMRs of the imprinted genes demonstrated that DNA demethylation occurred in the day-11.5 PGCs (*27*). Although DNA demethylation in day-10.5 PGCs was observed in only some of the DMRs, this population increased in day-11.5 PGCs, albeit to a different extent for each imprinted gene. Finally, DNA methylation was lost in all three regions in day-12.5 PGCs (*27*). Therefore, the erasure process of genomic imprinting occurs around day 10.5, at which stage the migrating PGCs reach and start to enter the genital ridges (Figs. 1 and 2) (*28*). Immigration to the genital ridges is completed within one day. Given that the demethylation process starts just after the PGCs enter the genital ridges, this clearly explains the differential DNA methylation patterns seen in the day-10.5 to -11.5 PGCs and the expression profiles of imprinted genes in the day-11.5 PGC clones. Interestingly, the kinetics of erasure differed among imprinted genes that showed biallelic expression, while non-expressed imprinted genes appeared to be synchronized, which suggests different erasing mechanisms. Thus, the characterization of the processes of genomic imprinting erasure, and of the establishment and maintenance of parental memory, provides novel insights into the regulation of genomic imprinting.

Rapid DNA demethylation during the erasing process suggests that active DNA demethylation occurs in PGCs as well as in male pronuclei just after fertilization (*29*) (Fig. 1). However, the biochemical process underlying DNA demethylation remains unclear, because demethylase activity has not been demonstrated in this system. However, it is now generally accepted that DNA methylation is involved in genomic imprinting. Dnmt1 is a maintenance-type DNA methyltransferase that recognizes hemimethylated DNA in replication forks and methylates the newly synthesized DNA strand. The loss of Dnmt1-mediated DNA methyltransferase activity changes the expression from monoallelic to biallelic, or abrogates the expression of imprinted genes (*30*). Therefore, it appears that Dnmt1 plays an essential function in maintaining parent-of-origin-specific memory in somatic cells (Fig. 1). Importantly, the expression profiles of imprinted genes in *Dnmt1* knockout (KO) mice were almost the same as those in day-12.5 PGC clones (Fig. 2), with the exception of the *Mash2* gene. *Mash2* expression was reported to be unaffected in *Dnmt1* KO mice (*31*, *32*), while its expression was clearly decreased in day-12.5 PGC clones (*27*), which suggests that the maintenance of *Mash2* imprinting differs from that of other genes.

1.5. Examples of deficient maternal imprinting in humans and mice—Recently, maternal imprinting was shown to be lacking in the oocytes of female *Dnmt3L* KO mice (*33*, *34*). When fertilized with normal sperm, the *Dnmt3L* KO oocytes showed early embryonic lethality around day 10.5. Interestingly, only the maternally imprinted *Peg* and *Meg* genes showed defective monoallelic expression (biallelic or null expression). In contrast, the paternally imprinted genes were not affected because normal male mice were used for mating. *Dnmt3L* is highly homologous to the *de novo* DNA methyltransferases *Dnmt3a* and *3b* (*35*), but lacks some essential domains for DNA methyltransferase, and thus lacks intrinsic enzymatic activity. It was suggested that *Dnmt3L* co-operated with *Dnmt3a* and *3b* to establish DNA methylation patterns in germ line cells, since a combination of *Dnmt3a*−/− and *Dnmt3b*+/− also showed a loss of maternal imprinting (*34*). These studies indicate that *de novo* methylation in germ cells plays an essential role in the establishment of maternal imprinting (Fig. 1).

A lack of maternal imprinting has been also demonstrated in the human biparental complete hydatidiform mole (biCHM) (*36*). Most CHMs arise from androgenetic development, but some are of biparental origin. In some biCHM patients that show repeated progression of hydatidiform moles, maternally imprinted *Pegs* and *Megs* display the expression profile of the default state. This indicates that oocytes lack the maternal imprinting mechanism. The expression profiles of the imprinted genes resemble those of *Dnmt3L* KO mice. It is highly possible that the gene responsible for biCHM collaborates with the Dnmt3L DNA methylation system, and plays an important role in maternal imprinting.

Taken together, these findings point to the existence of paternal and maternal imprinting mechanisms that regulate different sets of *Pegs* and *Megs*, thereby establishing paternal or maternal expression profiles in gametes or somatic cells. In addition to *Dnmt3L*, *Dnmt3a*, and *Dnmt3b*, other genetic factors (including the gene responsible for biCHM) that participate in maternal imprinting should be identified in future experiments. DNA recognition is necessary in both primary maternal and paternal imprinting and in *de novo* DNA methylation (*33–35*). Both Dnmt3L and the gene product that gives biCHM may have DNA recognition activities. Screening for DNA recognition factors will be important in elucidating the molecular mechanisms behind the establishment of parental genomic imprinting memories in both male and female germ cells.

1.6. Regulation of imprinted genes in somatic cells—How are the reciprocal *Pegs* and *Megs* expression patterns in somatic cells derived from the parental imprints that were established in the germ lines? The mechanism of imprinted gene expression in somatic cells has been studied extensively, and two regulation models have been proposed. It is well known that the imprinting control region upstream of *H19* regulates the reciprocal expression of both the paternal *Igf2* and the maternal *H19* genes (*37*); this type of regulation is attributed an 'insulator model' (*38*, *39*) (Fig. 3A). The *H19* transcript itself has no functional role and is dispensable (*40*), but specific

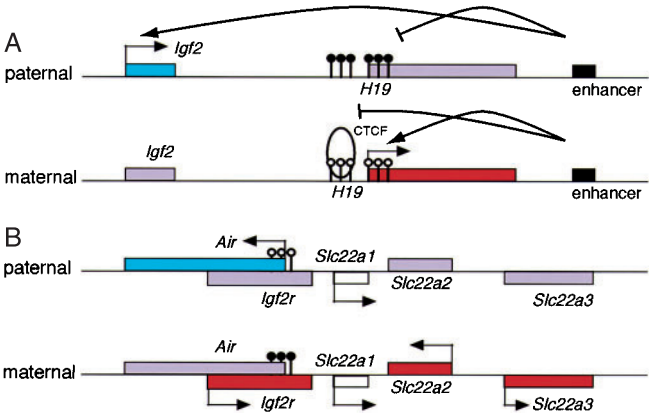

Fig. 3. **Two models of imprinting regulation in somatic cells.** (A) The insulator model. DMRs that lie upstream and in the promoter region directly regulate the expression of *H19*, and the specific binding of CTCF to the upstream primary DMR indirectly regulates the expression of *Igf2* by inhibiting the effect of downstream enhancer(s). Since CTCF binding is DNA methylation-sensitive, reciprocal expression of *H19* and *Igf2* occurs (*37*, *38*). Similar regulation by CTCF is observed for the mouse *Meg1/Grb10* (*40*). The two promoters of the mouse *Meg1/Grb10* are located in similar positions to those of *Igf2* and *H19* in this model. The primary DMR, which is DNA methylated in oocytes, overlaps with the second (downstream) promoter that regulates the paternally expressed transcript in the brain. On the other hand, the maternally expressed transcript is transcribed from the first (upstream) promoter by the secondary mechanism of CTCF-binding to the primary DMR. Recently, the contribution of another insulator protein (YY1) to *Peg3* regulation has been proposed (*87*). (B) The antisense model. The primary DMR in the mouse *Igf2r* region resides in the promoter region of the *Air* transcript, and regulates its expression directly. The expression of paternally expressed *Air* is essential for the repression of *Igf2r* and other maternally expressed imprinted genes (*Slc22a2* and *Slc22a3*) in the paternal allele, whereas *Air* is repressed and the other genes are expressed when DMR is methylated in the maternal allele (*45*). The *Slc22a1* gene in this region shows biallelic expression. The precise mechanism underlying this regulation remains unknown.

binding of CTCF (CCCTC-binding factor) to the control region that contains the DMR plays an important role in the reciprocal expression of *Igf2* and *H19* in this model (*38*, *39*). When the DMR is nonmethylated in the maternal alleles, *H19* expression occurs and *Igf2* repression is induced secondarily, by the binding of CTCF to this region, which results in the inhibition of the enhancer function in the downstream region. In contrast, when the DMR is methylated in the paternal alleles, *H19* is repressed and *Igf2* expression occurs, because CTCF binding is DNA methylation-sensitive (Fig. 3A). Recently, we demonstrated that a similar 'insulator model' applied to the mouse *Meg1/Grb10*. Interestingly, although *Meg1/Grb10* was originally identified as a maternally expressed gene (*17*), it shows paternal expression only in the brain (*41*, *42*). On the other hand, the human homologue *GRB10* is imprinted in the brain (paternal expression), but not in other tissues and organs, and shows an equal biallelic expression pattern (*43*, *44*). In order to elucidate the different imprinting regulation pathways in humans and mice and among different tissues, we compared the genomic sequences of these genes and examined their expression profiles in various tissues. In both species, the DMRs that overlapped with the second (downstream) promoter regions were already established in unfertilized eggs, and these DMRs directly regulated the paternal expression of mouse *Meg1/Grb10* and human *GRB10* in the brain. On the other hand, maternal expression of mouse *Meg1/Grb10* from the upstream promoter in all other tissues was regulated secondarily by CTCF binding, *via* a mechanism that is similar to that used in *H19-Igf2* regulation (Fig. 3A). However, since there is no CTCF-binding sequence in the human *GRB10*, the upstream promoter showed a biallelic expression pattern. This means that the upstream promoter of human *GRB10* is free from the imprinted regulation enforced by the primary DMR in the downstream promoter region. Thus, different imprinting expression patterns among different tissues may be explained by the differential usage of the two promoters, each of which shows paternal and maternal expression in mice, and paternal and biallelic expression in humans, respectively. Moreover, differential regulation among these species is explained by the presence of the mouse-specific CTCF-binding sequence, which results in secondary maternal expression from the upstream promoter in the mouse. These results indicate that a combination of genomic functional units, which include DMRs, promoters, insulator sequences, and enhancer sequences, is important for the establishment of expression profiles in somatic cells. Based on our understanding of the secondary mechanism, the expression of both *Pegs* and *Megs* may be explained by the existence of a single primary DMR in each imprinted region, whose establishment in the germ line depends on either paternal imprinting or maternal imprinting, as discussed above.

Another type of imprinted gene regulation in somatic cells is illustrated in the antisense model (Fig. 3B) (*45*). The paternally expressed non-coding *Air* transcript, which represents the antisense form of the maternally expressed *Igf2r* gene, is essential for the regulation of three reciprocally expressed imprinted genes, which include *Igf2r*, at the same locus. However, the underlying mechanism may prove to be rather complex, because the *Air* transcript overlaps only with *Igf2r* and not with the other target imprinted genes, *Slc22a2* and *Slc22a3* (Fig. 3B) (*45*). The production of double-stranded RNAs from the *Igf2r* and *Air* transcripts may function to silence gene expression by a mechanism that resembles RNA interference (RNAi), followed by subsequent inhibition of the surrounding region by an unknown mechanism. Elucidation of the precise mechanism will be required to attain a better understanding of the function of antisense transcripts in mammalian gene regulation.

In conclusion, the paternal or maternal imprint established in germ cells leads to the formation of the primary DMRs. In the somatic cell lineages, DNA methylation of the primary DMRs in each imprinted region directly silences some imprinted genes, and indirectly activates other imprinted genes by secondary mechanisms, such as those described in the insulator and antisense models. Thus, the entire regulation of genomic imprinting appears to have originated from a combination of primary and secondary mechanisms. The detailed analyses of these molecular mechanisms will be very important, not only to verify genomic imprinting as a mammal-spe-

Table 2. **The biochemical functions of imprinted genes.** The literature and genetic information relating to each gene are available on the website (*4*).

Signal transduction and cell cycle regulators	
Ligands	*Igf2*, *Ins2*, *Peg9/Dlk1*, *Ins1*
Receptors	*Obph1*, *Htr2a Igf2r*
G proteins	*Gnas*, *Gnasxl*
Others	*Nesp*, *p57KIP2/Cdkn1c*, *Peg12/Frat3*, *Ipl/Tssc3*, *Rasgrf1*, *Meg1/Grb10*, *Asb4*
Transcription factors and nuclear proteins	
Zinc finger	*Zim1*, *Peg3/Pw1*, *Zim3*, *Zpf264*, *Zfp127/Mkrn3*, *Zac1*
Others	*Magel2*, *Ndn*, *Mash2*, *Nap1l4*
Splicing factors	
	Snrpn, *U2af1- rs1*
Enzymes	
Ubiquitination	*Usp29*, *Ube3a*
Others	*Peg1/Mest*, *Dio3*
Channels and transporters	
	Kvlqt1, *Slc221l*, *Slc22a2*, *Slc22a3*, *Nnat*, *Ata3*
Surface antigens	
	Tapa1/Cd81
Structural genes	
	Sgce, *Dcn*
RNAs	
Antisense RNAs	*Nespas*, *Zfp127as/Mkrn3as*, *Ube3aas*, *Igf2as/Peg8*, *Kvlqt1-as/Lit1*, *Igf2ras/Air*
SnoRNAs	*MB11–13*, *MB11–52*, *MB11–85*, *MB11343*
X-inactivation	*Xist*, *Tsix*
Others	*Meg3/Gtl2*, *H19*, *IPW**, *PAR4**, *PAR5**, *PAR7**
Factors of unknown function	
	Snurf, *Tssc4*, *Impact*

*Human imprinted genes or transcripts.

cific mechanism but also to understand the complex genomic function of mammals. It should be noted that promoters, insulators, and enhancers are the fundamental genomic units in all living organisms. Therefore, the establishment of the DMR is an essential factor in the mammal-specific gene regulation system of genomic imprinting.

2. The biological significance of genomic imprinting

2.1. Biochemical functions of imprinted genes—Are there common biochemical and/or biological functions among imprinted genes that enable us to deduce the biological significance of genomic imprinting? To date, more than 60 imprinted genes have been isolated from the human and mouse genomes (*4–18*). Although some of these genes, such as *Igf2r/IGF2R*, *U2af-rs1/U2AF-RS1*, and *Impact/IMPACT*, are imprinted in mice but not in humans, most of the imprinted genes are conserved in both species.

As shown in Table 2, the biochemical functions of imprinted gene products are diverse, and include mediators of signal transduction and cell cycle regulation, transcription factors, enzymes, splicing factors, and structural proteins. Moreover, this collection encompasses substantial numbers of non-coding RNAs, some of which lack definitive functions. However, this may not be a unique character of imprinted genes, as it has been demonstrated recently that non-coding RNA is a major component of the transcriptome in the mouse genome (*46*). Small nucleolar RNAs (snoRNAs), which are located as clusters in two imprinted regions, may function in mRNA modification of unknown targets (*47*, *48*). The snoRNAs exist in introns of extra-long transcripts, and it is of interest to note that the important regions of these genes lie not in the *IPW*, *PAR1*, and *PAR5* exons in the PWS region, but in the introns that contain the snoRNA units. Non-coding antisense transcripts, such as *Ube3aas*, *Kvlqtas/Lit1*, and *Tsix*, presumably participate in the regulation of reciprocally expressed imprinted genes that have biochemical functions, as mentioned above for *Air/Igf2as* (*45*).

One of the important characteristics of imprinted genes is that they exist as members of imprinted gene clusters. The PWS/AS regions consist of six imprinted genes and one large transcript that contains several snoRNA units, and the BWS regions contain at least 12 imprinted genes. Generally, there are no apparent functional relationships among genes that are located in the same chromosomal regions. Therefore, it is reasonable to speculate that imprinted genes have diverse biochemical functions and lack common biochemical functions. What, then, can be said of their biological functions? Are there any functional relationships between the imprinted genes?

2.2. Conflict hypothesis—Of the many hypotheses that have been put forward to explain genomic imprinting, the 'conflict hypothesis' relates specifically to common biological functions among the imprinted genes (*49*). This hypothesis predicts that paternally expressed genes promote embryonic growth, while maternally expressed genes inhibit embryonic growth as a consequence of con-

flict between the paternal and maternal alleles during mammalian evolution. According to this hypothesis, the control of these growth-related genes by opposing factors is advantageous from both the paternal and maternal perspectives, with respect to long-term reproductive strategies. On the paternal side, there is evolutionary merit in creating larger progeny *via* the expression of growth-promoting genes, so that these progeny have a competitive advantage over those of other males. On the other hand, maternal expression of growth-repressive genes counters the paternal influence and conserves maternal resources for future pregnancies. Thus, the opposing gender pressures act as a driving force to establish the monoallelic expression system of genomic imprinting during mammalian evolution. Although this hypothesis is testable using a number of imprinted genes, it does not reveal much about the molecular evolution of this system.

It is evident that many imprinted genes (including both *Pegs* and *Megs*) have the expected functions. *Peg1/Mest* (putative hydrolase enzyme) (*15*, *50*), *Igf2* (fetal growth factor) (*6*), placenta-specific *Igf2* (*51*), *Peg3* (zinc-finger protein) (*16*, *52*), *Peg9/Dlk1* (delta-like 1 homologue to *Drosophila*) (*53*; J. Laborda, personal communication), *Rasgrf1* (Ras protein-specific guanine nucleotide releasing factor 1) (*54*), *GnasXl* (unknown function) (J. Peters and G. Kelsey, personal communication), and *Dio3* (thyroid hormone deiodinase type 3) (*55*) have been shown to function in growth promotion during the embryonic and/or neonatal periods. Deficiencies in maternally expressed genes, such as *Igf2r* (degradation of the Igf2 peptide) (*56*) and *Meg1/Grb10* (inhibition of signal transduction *via* insulin/insulin-like growth factor receptors) (A. Ward, personal communication), also promote embryonic growth, which indicates that these genes are inhibitory during development. However, it is also apparent that not all imprinted genes follow this hypothesis, especially those that have no apparent function (*H19*, *Snrpn*, *U2af1-rs1*), or that have unmatched phenotypes (*Mash2*, *p57Kip2*). It is also apparent that individual genes have different functions at different stages in development. Both *Peg1/Mest* and *Peg3* play essential roles in embryonal and placental growth, and they are required for maternal behavior in adult females (*50*, *52*).

The majority of imprinted regions show embryonic and/or neonatal growth effects, and placental abnormalities appear when the entire imprinted region becomes uniparental (*4*). However, several other defects, such as lethality at various developmental stages, morphological abnormalities, and behavioral or mental abnormalities, are also observed. The fact that the imprinted genes exist in gene clusters and are co-regulated by the same local mechanisms makes it difficult to test this hypothesis, because it is based on the effects of single genes rather than clusters of genes. Therefore, the hypothesis may need to be modified, as discussed below. Furthermore, recent findings suggest that some of the alternative hypotheses on the significance and origin of genomic imprinting merit re-consideration.

2.3. Prohibition of parthenogenetic development and protection from malignant trophoblast cell development—The biallelic expression of genes from males and females, which is the cornerstone of Mendelian genetics, is advantageous for individual animals because it enables them to avoid the debilitating effects of recessive mutations. Does parental-origin-specific monoallelic expression of a small subset of genes endow specific evolutionary advantages in mammalian development and growth that overcome the defects of recessive diseases?

As pointed out previously, monoallelic expression of some essential genes makes it impossible for mammals to develop parthenogenetically (*1*, *2*). Accidental or unexpected parthenogenesis in females is life-threatening and undesirable in nature, because food and environmental factors, such as temperature and climatic conditions, which are suitable for breeding pups, are seasonal. Therefore, the prohibition of parthenogenetic development is advantageous for mammalian reproduction (*57*). However, the existence of one or more of the *Peg* genes that are essential for development may be sufficient for this purpose, and *Megs* are not required for this hypothesis. As discussed above, the *Pegs* and *Megs* in each gene cluster are reciprocally regulated *via* the same mechanism, which explains the existence of both types of transcript (Fig. 3). However, this does not explain why so many imprinted genes exist. Genetically, this type of developmental system requires genetic contributions from both parents, and is evolutionarily advantageous in that it ensures species divergence by mixing genetic information. However, only mammals have adopted this system. In contrast, parthenogenetic development in many higher vertebrates, such as fish, amphibians, reptiles, and birds, occurs under both natural and artificial experimental conditions.

Another biological advantage of genomic imprinting is the absence of placental tissues during parthenogenetic development (*58*). Parthenogenetic mammals never develop to term and have negligible trophoblast cell expansion (*1*, *2*). Because placentas are infiltrative tissues that invade the maternal uterus, females can avoid developing malignant ovarian teratocarcinomas, even when they happen to undergo parthenogenetic conceptus (*58*).

These hypotheses suggest that there is some merit in genomic imprinting for mammalian development. However, this does not mean that genomic imprinting evolved to prohibit parthenogenetic development and/or invasive trophoblast development. Using experimental approaches, it is generally difficult to prove the hypothesis that genomic imprinting evolved for the acquisition of specific functional properties. However, if we were able to produce genetically engineer mice that developed parthenogenetically due to the regulation of the expression levels of some imprinted genes, we might be able to see whether these embryos and/or neonates conferred certain disadvantages on their mothers. Furthermore, the identification of imprinted genes that are essential for trophoblast cell invasion would make it possible to test the theory that biallelic gene expression causes undesirable changes in pregnant females. Nonetheless, continued systematic screening for imprinted genes is necessary for the verification of these hypotheses.

2.4. Genomic imprinting as a by-product of cellular defense against exogenous DNA—Another hypothesis has been put forward that suggests that genomic imprinting arose as a side-effect of cellular defense mechanisms

against exogenous DNA (the defense mechanism hypothesis) (*59*). According to this hypothesis, genomic imprinting originated as a by-product of the system through which the mammalian genome represses exogenous DNA sequences using DNA methylation. Retroviral sequences are extensively methylated immediately after they integrate into the mammalian genome. Furthermore, DNA methylation of several retrotransposon sequences, such as *L1* and *IAP*, is known to occur during mammalian development, and is completed at birth (*60*). Therefore, retrotransposon repression is almost complete in somatic cell lineages, although it is not clear that such retrotransposons are of exogenous origin. Interestingly, retrotransposons show high levels of expression in pre-implantation embryos and have monoallelic expression status, since they are differentially methylated in sperms and eggs. Since these features resemble those of the imprinted genes, common regulatory pathways may exist for the repression of retrotransposon DNA and genomic imprinting (*60*). However, the existence of DNA methylation and retrotransposons is not unique to mammals, since these systems occur commonly in vertebrates. Therefore, this hypothesis does not explain why genomic imprinting occurs exclusively in mammals.

Recently, we identified a retrotransposon-derived imprinted gene, *PEG10*, on human chromosome 7q21 (*61*). Another sushi-ichi, retrotransposon-derived, imprinted gene (*PEG11*) has also been demonstrated in a different imprinted region (*62*; Wagatsuma, unpublished data). Both the *PEG10* and *PEG11* genes have putative protein-coding sequences that correspond to the retroviral *gag* and *pol,* the latter of which is truncated. The locations of both these genes are conserved among mammals. Therefore, it seems likely that the integration of these retrotransposons occurred before mammalian divergence. It is interesting to test a new hypothesis that the integration of these genes is essential for establishing the imprinting state of the integrated regions (the retrotransposon insertion hypothesis). If this hypothesis is true, it lends strong support to the defense mechanism hypothesis, which states that imprinting is a consequence of defense against foreign DNA. Extensive studies are underway on *de novo* DNA methylation systems and the mechanisms of genomic imprinting, and we believe that these hypotheses will be testable at the molecular level in the near future.

2.5. Salvation of the developmental system by reciprocal expression of Pegs and Megs—A different view of the biological importance of genomic imprinting emerges from the proposed regulation mechanism of imprinted genes (discussed in Chapter 1). As shown in Table 1, approximately half of the imprinted genes (maternally imprinted *Megs* and paternally imprinted *Pegs*) are not expressed unless the DMRs are methylated and their reciprocally expressed imprinted genes (maternally imprinted *Pegs* and paternally imprinted *Megs*) are silenced (Table 1, Fig. 2). For example, the paternally expressed *Igf2* gene is induced only when it is paternally imprinted (methylated) and the maternal expression of *H19* is repressed (Fig. 3A), and the maternally expressed *Igf2r* gene is induced only when it is maternally imprinted (methylated) and the paternal expression of *Air* is repressed (Fig. 3B). The existence of the *Igf2* and *Igf2r* genes may be the key as to why genomic imprinting is essential and conserved in mammals, since these genes must be imprinted in one or other of the parental germ cells, otherwise the genes are never expressed in the somatic cell lineages during development and growth.

If both alleles are imprinted, the remaining imprinted genes lose their expression. Some of the *Pegs* (*Peg1/Mest, Peg3, Necdin, Peg9/Dlk1, etc.*) (*50*, *52*, *53*, *63*, *64*) and *Megs* (*Igf2r, p57Kip2, Mash2, etc.*) (*65–68*), which are under the influence of maternal imprinting, play essential or important roles in development and growth. This indicates that the current mammalian developmental system requires the expression of all these important *Pegs* and *Megs*. Therefore, we assume that the reciprocal expression system of *Pegs* and *Megs* was originally one of limited options for rescuing the mammalian developmental system from a potentially catastrophic situation, in which the expression of either half of the imprinted genes was lost. For this purpose, it was necessary to imprint either of the parental alleles, so as to produce different expression patterns in the paternal and maternal alleles of each imprinted region (the complementation hypothesis). Thus, the present genomic imprinting system, in which paternal and maternal imprints regulate different sets of imprinted genes, was established.

Our complementation hypothesis insists that monoallelic expression of imprinted genes is an inevitable consequence of mammalian evolution. Once the genomic imprinting system was adopted, mammals were dependent upon it. This explains why genomic imprinting is conserved in mammals and is essential for mammalian development and growth, because loss of the genomic imprinting system causes the silencing of many important genes. However, this does not reveal the origin of genomic imprinting; it only indicates how this system may have emerged. Although genomic imprinting is a mammalian-specific gene regulation mechanism, many of the imprinted genes are conserved in other vertebrate species. Therefore, comparative genomics of the imprinted regions in mammals and the corresponding regions in other vertebrates may elucidate evolutionary events in the mammalian genome. Thus, the molecular mechanism by which genomic imprinting originated in the course of mammalian evolution should provide clues to the biological significance of genomic imprinting.

2.6. A novel hypothesis regarding the placental expression of imprinted genes—Viviparity is one of the most important characteristics of mammals (more precisely, of marsupial and eutherian animals) and may be an important factor for genomic imprinting (the placenta hypothesis; *69*). Recently, the existence of genomic imprinting was demonstrated in the marsupial opossum species, with the paternal and maternal expression of *Igf2* and *Igf2r*, respectively (*70*, *71*). Marsupials have incomplete placentas that consist of yolk sac membranes, in contrast to the fully functional chorionic placentas in eutherian animals. However, to date, there is no evidence that imprinted genes exist in monotremes (*72*), egg-laying mammals, or birds (*70*, *73*). These facts underscore the significant relationship between placental formation and genomic imprinting in mammals, and strongly support this hypothesis.

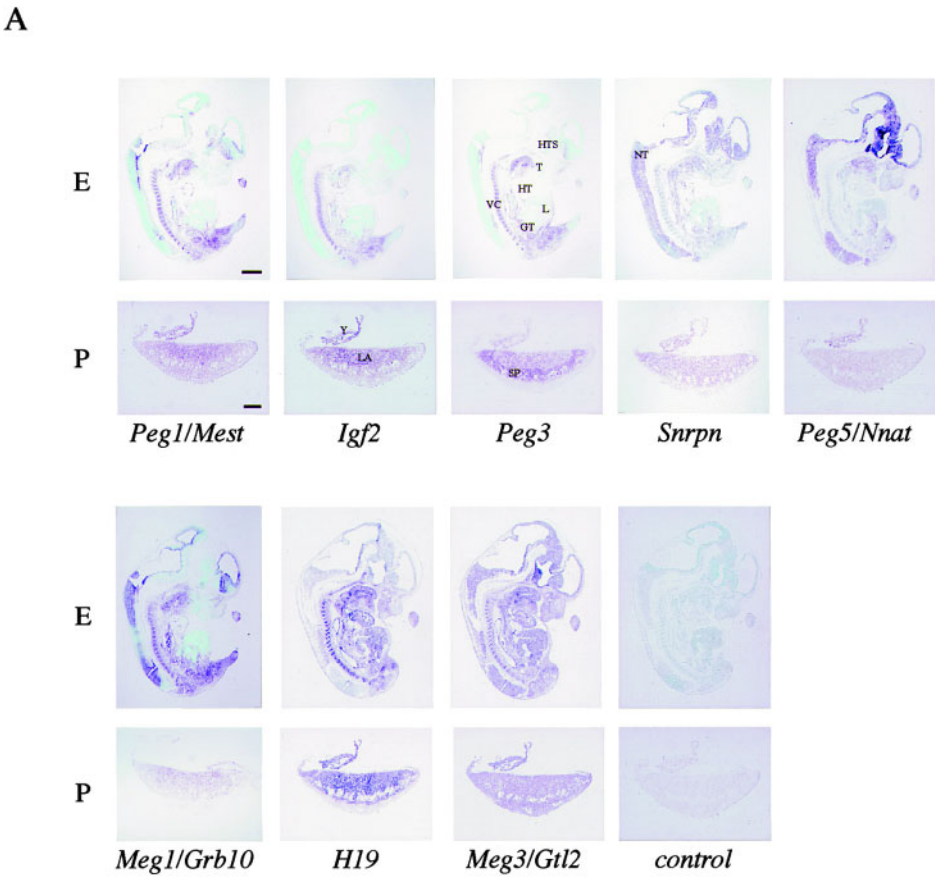

Fig. 4. **Expression patterns of imprinted genes in embryos and placentas, as revealed by in situ hybridization.** (A) Gene expression profiles in day-12.5 embryos and placentas. In the upper column: E, embryos; GT, gut; HT, heart; HTS, hypothalamus; L, liver; NT, neural tube; T, tongue; VC, vertebral column. In the lower column: P, placentas; SP, spongiotrophoblast; LA, labyrinth; Y, yolk sac. *Peg5/Nnat* is expressed specifically in the chorioallantoic plate and yolk sac. The expression of *Peg7* (placental *Igf2*) and *Igf2as/Peg8* is observed in placentas, although not at high levels at this stage (data not shown). (B) The gene expression profiles of whole day-9.5 embryos that are surrounded by decidua and the uterine wall. AL, allantois; DE, decidua; EM, embryo; G, giant trophoblast; PL, placenta; U, uterine wall. Placental expression of *Peg7* (placental *Igf2*) and *Igf2as/Peg8* is clearly observed, particularly for *Igf2as/Peg8* in the giant trophoblast. The dark colors observed in the embryos are due to the methyl green counter-stain, and do not represent authentic signals from the NBT/BCIP color reaction that was used for the *in situ* hybridization experiments.

As described above, each of the imprinted genes has a different biochemical function and is expressed in a different tissue and organ during embryonic development and growth. However, we observed that the imprinted genes that were isolated during our systematic screening showed common placental expression, in spite of their having different expression sites in embryos (*74*). The results of the *in situ* hybridization experiment are shown in Fig. 4A. The expression of *Meg3/Gtl2* was observed in almost all the day-12.5 embryonic tissues (*75*). The *Peg1/Mest*, *Igf2*, *Peg3*, and *H19* genes were highly expressed in mesodermal tissues at this stage (*6*, *15*, *16*), whereas the *Snrpn* and *Peg5/Nnat* genes were highly expressed in ectodermal tissues (*76*, *77*). The *Meg1/Grb10* gene was expressed in both the mesodermal tissues and some parts of the ectodermal tissues (*17*). Interestingly, *Peg7* (placental-specific *Igf2*) and *Igf2as/Peg8* showed no expression in the embryonic components, but were expressed in the placenta (*78*, *79*). The expression profiles of *Peg7* and *Igf2as/Peg8* in the extra-embryonic tissues were more evident at day 9.5 of gestation (Fig. 4B). Importantly, all of these genes were expressed in extra-embryonic cells (trophoblast and yolk sac cells), with the exception of *Peg5/Nnat*, which was expressed mainly in the chorioallantoic plate and in certain yolk sac cells. Increasing numbers of imprinted genes have been reported to show placental (extra-embryonic tissues) expression during development.

From these results, we postulate that imprinted genes are controlled so as to bring about their expression in placental tissues (the novel placenta hypothesis). The potential relationship between the placenta formation and genomic imprinting in mammals has been proposed previously as the 'former placenta hypothesis' (*69*), although no evidence was provided for a molecular mechanism. However, it is possible that placental expression of

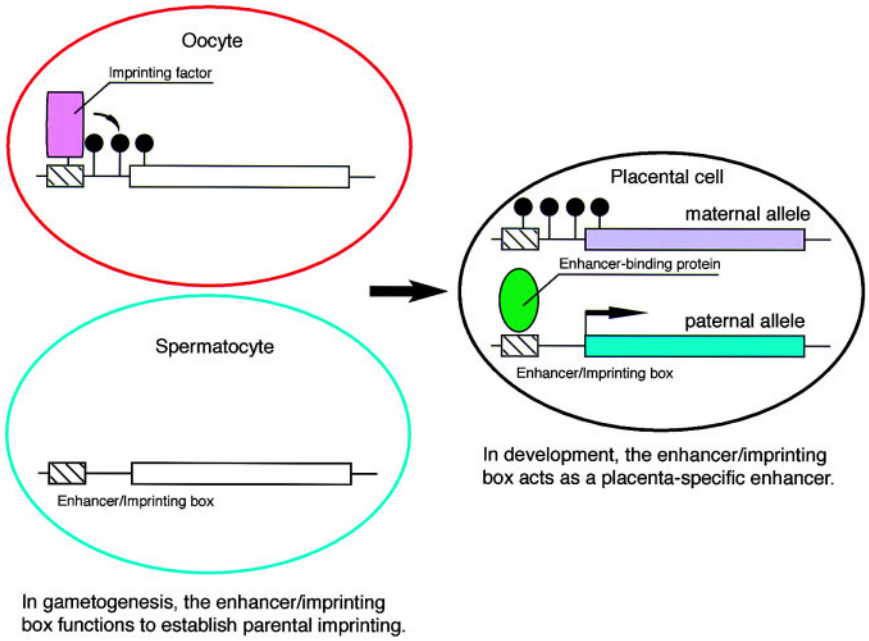

Fig. 5. **A novel hypothesis that links placental development and mammalian evolution.** Since most imprinted genes show placental expression, it is assumed that imprinted genes are regulated to ensure appropriate expression in the placental tissue, which includes the yolk sac. The putative enhancer and imprinting box sequences (hatched boxes) are *cis*-regulatory elements, which may function as imprinting control sequences in gametogenesis to produce genomic imprinting memories (left) and placental enhancers during development (right). In this diagram, maternal imprinting is considered. The imprinting factor functions in the formation of the primary DMR, and it is maintained in the somatic cell lineages. Different *cis*-regulatory elements and additional factors that recognize these elements are required to explain both the paternal and maternal imprinting mechanisms. The principal biological significance of genomic imprinting in mammalian development and evolution may lie in the promotion of expression of essential genes, which make it possible to form mammalian-specific organs, such as placentas.

imprinted genes represents a particular genetic change that occurred during mammalian evolution, which enabled the ancestral mammal to form placental structures. For example, genetic change(s) to some DNA-binding protein(s) may have occurred. This could have led to an acquired ability to bind to a common (or similar) DNA sequence (identical to the putative imprinting box sequences) on imprinted genes. Thus, the expression of imprinted genes would be induced in novel placental tissues during development, while the same recognition system would function in gametogenesis and cause genomic imprinting (Fig. 5).

The aforementioned alteration of the gene regulation network would have been a random process. Therefore, a variety of genes would have come under the control of genomic imprinting, including those required for placental formation. It is conceivable that only a proportion of the genes would be actually involved in, and essential for, placental function, although all of the imprinted genes would show placental expression. Thus, important genes of this type could affect embryonal and postnatal growth. For example, *Mash2* plays an essential role in placental development by regulating the growth of the spongiotrophoblast tissue (*68*). In addition, substantial numbers of imprinted genes, which include *Peg1/Mest*, *Igf2*, *Peg3*, *Meg1/Grb10*, *p57Kip2*, *Ipl*, and placental *Igf2* (*Peg7*), are known to function in placental growth and function (*6*, *50–52*, *80–82*). Imprinted X-inactivation, which is regulated by the paternally expressed *Xist* gene (*83*) that occurs exclusively on the paternally derived X chromosomes in eutherian extra-embryonic tissues (*84*), may also be important for placental development in females. Therefore, genomic imprinting may have facilitated placental acquisition during evolution by promoting the expression of a variety of placental genes.

Our novel placenta hypothesis predicts that the acquisition of placental tissues in the mammalian developmental system is the *sine qua non* of genomic imprinting, because it changed the gene regulatory system that was associated with the mutation of some DNA recognition factor(s). From the evolutionary and biological standpoints, we propose that one of the important features of genomic imprinting is the placental expression of imprinted genes, although this hypothesis does not necessitate monoallelic expression *per se*. Further studies of genomic imprinting may give us insights into the crossovers between the monoallelic and placental expression pathways of imprinted genes.

Both of our hypotheses, the complementation hypothesis and the novel placenta hypothesis, take a definitive stand on the essential nature of genomic imprinting in mammalian development and growth, albeit from different points of view. These hypotheses refer to different phases of genomic imprinting: the former to maintenance, and the latter to establishment of the system. Therefore, these hypotheses are not mutually exclusive, and may corroborate each other, although a unified theory is currently lacking. Our hypotheses may also be partly compatible with the previously proposed hypotheses. For example, although the defense mechanism and retrotransposon insertion hypotheses do not state the importance of genomic imprinting, they could be linked with our two hypotheses with regard to trigger or recognition sequences for genomic imprinting. We also think that our two hypotheses are partially compatible with the "conflict hypothesis." Once the genomic imprinting mechanism was established, the imprinted genes that followed the "conflict hypothesis" were more likely to be conserved among mammalian species because they conferred evolutionary merit. We believe that the "conflict hypothesis" may be important in this type of selection process, rather than in the establishment of monoallelic expression of imprinted genes, as was proposed originally. Hopefully, the accumulation of novel experimental data on the molecular mechanism of genomic imprinting

will lead to a unified theory that is based on the various hypotheses described here.

The monoallelic expression of autosomal genes has apparent disadvantages. However, genomic imprinting may play evolutionarily essential roles in the establishment of mammals, and remain indispensable for mammalian development and growth. As discussed in the first section, the regulatory system for genomic imprinting is being elucidated. Studies on the molecular mechanisms for establishing the parental primary imprint in germ cell lines and parent-of-origin-specific expression patterns in somatic cells are progressing rapidly. These investigations should provide us with important clues as to how these processes evolved in mammals. It will then be possible to consider the biological significance of genomic imprinting based on how imprinting affects the biological characteristics of mammals.

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