REVIEW

Genomic imprinting and its relevance to congenital disease, infertility, molar pregnancy and induced pluripotent stem cell

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Genomic imprinting is an epigenetic gene-marking phenomenon that occurs in the germline, whereby genes are expressed from only one of the two parental copies in embryos and adults. Imprinting is essential for normal mammalian development and its disruption can cause various developmental defects and diseases. The process of imprinting in the germline involves DNA methylation of the imprint control regions (ICRs), and resulting parental-specific methylation imprints are maintained in the zygote and act as the marks controlling imprinted gene expression. Recent studies in mice have revealed new factors involved in imprint establishment during gametogenesis and maintenance during early development. Clinical studies have identified cases of imprinting disorders where involvement of factors shared by multiple ICRs for establishment or maintenance is suspected. These include Beckwith-Wiedemann syndrome, transient neonatal diabetes, Silver-Russell syndrome and others. More severe disruptions can lead to recurrent molar pregnancy, miscarriage or infertility. Imprinting defects may also occur during assisted reproductive technology or cell reprogramming. In this review, we summarize our current knowledge on the mechanisms of imprint establishment and maintenance, and discuss the relationship with various human disorders.

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OVERVIEW OF GENOMIC IMPRINTING

Unlike most autosomal genes, which are expressed biallelically, a certain number of genes in mammals show highly biased or even monoallelic expression dependent on parental origin. The unique expression patterns are brought about by differences in epigenetic marks between the parental copies, which are established in the parental germline. This germline-specific gene-marking phenomenon is called genomic imprinting. Imprinting was first discovered by embryological experiments in mice, which showed that normal development requires both a paternal and a maternal genome.^{1,2} Almost at the same time, geneticists found by making uniparental disomies that certain mouse chromosome regions have differential activity depending on their parental origin.³ These results clearly pointed to the existence of imprinting phenomenon in mammals, and the first imprinted gene *Igf2r* was identified in 1991.⁴ The number of imprinted genes identified so far is around 100 in both humans and mice, but a recent study suggested that this may expand to over 1000.⁵

DNA methylation is a critical component of the imprinting mechanism,⁶ and allele-specific methylation is often found in imprinted gene regions (differentially methylated regions or DMRs).⁷ There are two DMR classes: primary DMRs and secondary DMRs. The primary or

germline DMRs acquire methylation in the germline (Figure 1) and often identical with imprint control regions (ICRs). The ICRs regulate expression of one or more proximal genes (imprinted genes often form clusters) through complex mechanisms involving non-coding RNAs, histone modifications, insulators and a higher order chromatin structure.⁸ Many ICRs have been identified and characterized by targeted deletion in mice, which often results in dysregulation of the entire imprinted cluster.^{9–15} Such mouse models often show phenotypes reminiscent of human diseases, and are useful for understanding their molecular mechanisms, which we will discuss later. The secondary or somatic DMRs acquire methylation after fertilization under the control of nearby ICRs.⁷ Once established in either gametes, the methylation imprint at an ICR is passed to the zygote and stably maintained in somatic lineages throughout its life (Figure 1).¹⁶ However, the differential methylation is erased in primordial germ cells in each generation before the new imprints are set.^{17–19}

THE MECHANISM OF IMPRINT ESTABLISHMENT IN THE GERMLINE

The first step of the imprinting cycle, imprint establishment, occurs in male and female gametogenesis. Imprint establishment takes place in

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Figure 1 Chronology of genomic imprinting and various factors involved in the two major steps, establishment and maintenance. The steps affected in infertility, molar pregnancies and miscarriage, and imprinting disorders are also shown.

growing oocytes in females and prospermatogonia in males. Oocyte growth occurs in the postnatal ovary, and growing oocytes are in the diplotene stage of meiotic prophase I. Prospermatogonia are G1-arrested male germ cells in the fetal testis, which eventually give rise to spermatogonia after birth. Below we summarize the recent advancement in studies on the mechanisms of imprint establishment in mice.

It has been shown by gene knockout experiments that the *de novo* DNA methyltransferase essential for imprint establishment is Dnmt3a in both male and female germlines (Figure 1).^{20–22} This enzyme forms a complex with the structurally related, germ-cell-specific cofactor Dnmt3L, which enhances the catalytic activity of Dnmt3a and is important for imprint establishment (Figure 1).^{21,23–25} The other *de novo* methyltransferase Dnmt3b is not involved, except at one particular ICR (see later). Thus, the key proteins in the imprint establishment machinery have been identified, but how they are recruited to specific targets is less clear.

Factors and features identified so far as specificity determinants for imprinting in oocytes include: (1) transcription traversing ICRs (Figure 1),²⁶ (2) unmethylated lysine-4 of histone H3 (H3K4),²⁷ (3) 10-bp CpG spacing,²⁸ (4) a histone H3K4 demethylase Kdm1b (Figure 1)²⁹ and (5) a Krüppel-associated box (KRAB) zinc finger protein Zfp57 (Figure 1).³⁰ Among these, the first three seem common to all oocyte-methylated ICRs and could be prerequisites for imprint establishment. Transcription through ICRs may make the chromatin more accessible by the Dnmt3a–Dnmt3L complex;²⁶ unmethylated, but not methylated, H3K4 is the high-affinity binding target of Dnmt3L;²⁷ two CpG sites located 10-bp apart fit very well with the catalytic centers of the heterotetrameric Dnmt3a–Dnmt3L complex.²⁸ However, these features can be found elsewhere in the genome and are

not specific to ICRs. By contrast, Kdm1b acts only on a subset of ICRs (for example, ICRs at *Mest*, *Grb10*, *Plagl1* and *Impact* loci),²⁹ although demethylation of H3K4 by this enzyme is consistent with the preference of Dnmt3L for unmethylated H3K4. The involvement of Zfp57 appears to be more restricted, and so far the *Snrpn* ICR is its only known target in oocytes, although it is involved in imprint maintenance at multiple ICRs after fertilization (see later).³⁰ Therefore, the process of specificity determination seems complicated, and perhaps combinations of common and ICR-specific factors may eventually produce the specificity. An alternative view is that there is no specific targeting of methylation to DMRs; instead, they represent a subset of CpG islands that acquire methylation in oocytes in response to general mechanisms of methylation.³¹

We recently found that methylation of the *Rasgrf1* ICR in prospermatogonia involves the PIWI-interacting RNA machinery (Figure 1).³² Evidence suggests that recruitment of the Dnmt3a–Dnmt3L complex requires an interaction between the non-coding RNA traversing the ICR and PIWI-interacting RNAs complementary to this RNA. So far, this is the only ICR involving the PIWI-interacting RNA pathway, and it is interesting that the involvement of Dnmt3b is also unique to this ICR.²¹ At the *H19* ICR, CTCFL (also known as BORIS) and a protein arginine methyltransferase Prmt7 appear to have roles in methylation imprint establishment in prospermatogonia (Figure 1).³³ CTCFL is a paralog of the insulator protein CTCF and highly expressed in the male germline. Recently, it was found that imprinted loci retain histones in mature sperm, raising the possibility that histone modification can be an imprint.³⁴ Notably, H3K4me3 is enriched at regions with no methylation in sperm, suggesting that this mark might protect the oocyte-methylated ICRs from methylation in the male germline.^{34,35}

IMPRINT MAINTENANCE IN EARLY EMBRYOS

The methylation imprints established in the parental germline should be protected from the genome-wide reprogramming including global DNA demethylation during early embryogenesis. In preimplantation embryos, the methylation imprints at ICRs are first copied by the activity of the oocyte-stored form of the maintenance DNA methyltransferase Dnmt1o and then by zygotically expressed somatic form Dnmt1s (Figure 1).³⁶ Interestingly, a recent study identified over a thousand CpG islands methylated by the Dnmt3a–Dnmt3L complex in oocytes.³⁷ Most of them are thought not to relate to imprinting and lose methylation at least partially during preimplantation development. This indicates the existence of mechanisms that recruit protection factors and/or maintenance enzymes specifically to ICRs. Dppa3 (also known as Pgc7 or Stella) is one protein that protects the maternal imprints from demethylation (Figure 1).³⁸ It likely acts through protection against hydroxylation of 5-methylcytosine by the Tet3 protein.³⁹ 5-hydroxymethylcytosine is not recognized by Dnmt1 and thus, without this protection, maternal imprints can be subject to replication-dependent passive demethylation. Zfp57 is an oocyte-derived maternal factor involved in preimplantation maintenance of both paternal and maternal imprints at multiple loci such as the paternally methylated intergenic ICR (IG-DMR) within the *Dlk1-Meg3* locus (*Meg3* is also known as *Gtl2*) and the maternally methylated *Mest*, *Peg3* and *Snrpn* ICRs (Figure 1).³⁰ However, there are ICRs that are not affected in *Zfp57* mutants (for example, *H19* and *Igf2r* ICRs).³⁰ The mechanistic detail of this locus-specific protection is still lacking.

CHILDHOOD DISEASES ASSOCIATED WITH IMPRINT ESTABLISHMENT OR EARLY MAINTENANCE

Imprinted genes have key roles in growth and development, and imprinting disorders are typically accompanied by growth-related symptoms.⁴⁰ Causes of imprinting disorders range from chromosomal and genetic abnormalities to epigenetic abnormalities and one imprinting disorder can result from a variety of molecular mechanisms. Chromosomal and genetic causes include uniparental disomies, chromosomal deletion, duplication, translocation, inversion and point mutation, all of which change the dosage of normal imprinted gene product(s). Epigenetic abnormality is mostly aberrant hypermethylation/hypomethylation at ICRs. We here focus on defects in establishment or early maintenance of imprints.

Beckwith-Wiedemann syndrome (BWS) is an overgrowth disorder characterized by macrosomia, macroglossia, organomegaly, omphalocele, neonatal hypoglycemia, ear creases or ear pits, and predisposition to childhood tumor. The chromosome region responsible for BWS is 11p15.5, which contains a large cluster of imprinted genes regulated by two ICRs: the *KCNQ1OT1* ICR (or KvDMR1) is located in the 5' region of *KCNQ1OT1* and maternally methylated, and the *H19* ICR is located between *H19* and *IGF2* and is paternally methylated (Figure 2).⁴¹ A variety of genetic defects have been identified in BWS patients, such as paternal uniparental disomies, deletion, duplication, translocation and mutations in *CDKN1C*. However, hypomethylation of the *KCNQ1OT1* ICR is the most frequent (50%) epigenetic change in BWS, and hypermethylation of the *H19* ICR is another (5%) (Table 1).⁴¹ Notably, it was reported that 20–24% of BWS cases are hypomethylated at multiple maternally imprinted loci other than the *KCNQ1OT1* ICR (Table 1).^{42–44} Moreover, a homozygous mutation in a nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing (NLRP) family gene, *NLRP2* was found in a mother of two BWS children with the *KCNQ1OT1* ICR hypomethylation, and one of the patients showed

a loss of methylation at another maternally methylated *MEST* ICR.⁴⁵ This implies a role for NLRP2 in the establishment and/or maintenance of methylation imprints at multiple ICRs.

Silver-Russell syndrome shows prenatal and postnatal growth retardation with heterogeneous clinical manifestations that are basically opposite to what is observed in BWS. A number of chromosomes and potential candidate genes are suggested to be associated with the disease, but a large proportion of Silver-Russell syndrome is linked to the same genomic region as BWS (11p15.5) and maternal uniparental disomies is one major cause (Figure 2).⁴⁶ Epigenetic abnormalities are detected in up to 35–65% of patients, the most frequent one being hypomethylation of the *H19* ICR (Table 1).^{46–52} Like BWS cases, a proportion of patients with Silver-Russell syndrome show hypomethylation at multiple ICRs (Table 1).^{43,53} Moreover, over two-third of such patients have hypomethylation not only at the maternally methylated ICRs but also at another paternally methylated ICR at the *DLK1-MEG3* locus (IG-DMR) (Table 1).⁴³ This suggests that the primary defect probably affects post-fertilization maintenance. However, the causative gene responsible for this phenotype has not been identified.

Transient neonatal diabetes mellitus type 1 is a rare type of diabetes with hyperglycemia manifested within the first 6 months of life. This is caused by overexpression of the imprinted genes *PLAGL1* and *HYMAI* in chromosome region 6q24. About 20% of patients with transient neonatal diabetes mellitus type 1 show loss of methylation at the *PLAGL1/HYMAI* ICR, which is normally maternally methylated (Figure 2 and Table 1).^{54,55} Over 50% of these patients also show hypomethylation at other maternally methylated ICRs across the genome, which was recently found to be associated with *ZFP57* mutations (Table 1).⁵⁴ The phenotype can be explained by defects in either establishment or maintenance of methylation imprints but, as noted above, studies on *Zfp57* knock-out mice showed that its predominant role is in maintenance after fertilization.³⁰ However, it is important to note that some individuals with hypomethylation at multiple ICRs carry no mutation in *ZFP57*, indicating the existence of other factors.

Pseudohypoparathyroidism type Ib, an endocrine disorder characterized by renal resistance to parathyroid hormone, is associated with loss of methylation at a maternally methylated ICR located at *GNAS* exon A/B in chromosome region 20q13.3 (Figure 2 and Table 1).^{56–59} A remarkable finding with this disorder is that genetic mutations underlying the loss of methylation were initially mapped ≥ 56 kb upstream of the exon A/B ICR, indicating the presence of a long-range, *cis*-acting element.⁵⁶ Furthermore, heterozygous microdeletions at *STX16*, located approximately 220 kb upstream of the *GNAS* exon A/B ICR, were also found in most cases of autosomal dominant form of pseudohypoparathyroidism type Ib.^{57,60–62} *STX16* appears to be a non-imprinted gene, but only maternal transmission of these microdeletions leads to this disorder, suggesting the existence of another long-range *cis*-acting element. Another study identified maternally transmitted deletions of an upstream exon of the *NESP55* transcript within the *GNAS* locus in two families, which were associated with methylation defects at all downstream *GNAS* ICRs located at exons AS1, XL and A/B (Figure 2 and Table 1).⁶³ At least some of these deletions could affect transcription of *NESP55* in oocytes, which is presumed to proceed through AS1, XL and exon A/B. In light of the study on the mouse *Gnas* locus,²⁶ it is conceivable that the absence of *NESP55* transcription leads to a failure in imprint establishment.

Angelman syndrome (AS) is a neurodevelopmental disorder caused by a deficiency of a maternally expressed imprinted gene *UBE3A*.^{64,65} AS is usually associated with genetic abnormalities at an imprinted cluster containing *UBE3A* within 15q11–q13, and approximately 2–4% of the patients show epigenetic defects at this region, which

Figure 2 Schematic representation of the human-imprinted loci associated with diseases (not drawn to scale). **(a)** The *KCNQ1* and *H19* loci for Beckwith-Wiedemann syndrome (BWS) and Silver-Russell syndrome (SRS) in 11p15.5. **(b)** The *PLAGL1/HYMAI* locus for transient neonatal diabetes mellitus type 1 (TNDM1) in 6q24. *PLAGL1* has an upstream promoter, which is biallelically expressed. The ICR overlaps with a promoter shared by *PLAGL1* and *HYMAI*. **(c)** The *GNAS* locus and *STX16* for pseudohypoparathyroidism type Ib (PHP-Ib) in 20q13.3. **(d)** The *SNRPN* locus for Angelman syndrome (AS) and Prader-Willi syndrome (PWS) in 15q11-q13.

include loss of methylation at the ICR at an imprinted bicistronic transcript encoding small nuclear ribonucleoprotein N (*SNRPN*) and *SNRPN* upstream reading frame (*SNURF*) (referred to as *SNRPN*) (Figure 2 and Table 1).^{66–68} Among those, 92% are thought to have epigenetic mutations occurring in either oocytes or early embryos.⁶⁶ Maternally transmitted deletions of a region located 35 kb upstream of the *SNRPN* ICR, along with loss of methylation, have been identified in AS patients, which led to the discovery of the AS-imprinting center.^{69,70} The *SNRPN* gene has a number of alternative transcripts, and AS-imprinting center overlaps with exons of such transcripts.^{71,72} Therefore, one possible explanation for some cases would be that the deletions affect oocyte-specific alternative transcripts traversing the *SNRPN* ICR, and this results in a failure in imprint establishment. Prader-Willi syndrome (PWS) is another neurogenetic disorder that results from the abnormalities of the same imprinted cluster as AS. In contrast to AS, chromosomal deletions found in PWS are always on the paternal chromosome. A *de novo* interstitial deletion affecting the

entire imprinted domain, and a maternal uniparental disomy are the major causes of PWS (~70% and ~25–30%, respectively), and about 1–3% of PWS is caused by abnormal imprinting.^{40,66} A small proportion of PWS patients with an imprinting defect carry a deletion at the PWS-imprinting center that lies at the promoter/exon 1 of *SNRPN* (Figure 2).⁶⁶ However, most imprinting defects represent primary epimutations derived from the paternal grandmother. This suggests that, unlike other diseases, incorrect erasure of the imprint in the paternal primordial germ cells might have resulted in inheritance of a chromosome carrying an imprinting defect in such patients,⁶⁶ but the factor involved in such defects is not clear.

MOLAR PREGNANCY, INFERTILITY, ASSISTED REPRODUCTIVE TECHNOLOGY (ART) AND INDUCED PLURIPOTENT STEM (IPS) CELLS

More severe defects in imprint establishment in the germline are probably incompatible with life and lead to infertility or miscarriage.

Table 1 Methylation defects at ICRs reported in imprinting disease, molar pregnancy, infertility and iPS cells

Disease/condition	Gene locus for ICR showing methylation defects		Candidate factor involved in methylation defects at ICR	Reference
	Maternally methylated	Paternally methylated		
BWS ^a	<i>PLAGL1/HYMAI, IGF2R, MEST, GRB10, KCNQ1OT1, SNRPN, PEG3, GNAS</i> (AS1 and A/B ICRs)	<i>H19</i>	NLRP2	42–45
SRS ^a	<i>PLAGL1/HYMAI, IGF2R, MEST, KCNQ1OT1, SNRPN</i>	<i>H19, DLK1-MEG3</i> (IG-DMR)		43, 53
TNDM1 ^a	<i>PLAGL1/HYMAI, MEST, GRB10, KCNQ1OT1, PEG3, GNAS</i> (AS1 ICR)		ZFP57	54
PHP-Ib ^a	<i>GNAS</i> (AS1, XL and A/B ICRs)		Genomic deletion of <i>NESP55</i> or <i>STX16</i>	56–58, 60–63
AS/PWS	<i>SNRPN</i>		Genomic deletion of AS-IC (AS)	66–68
BiCHM ^a	<i>MEST, KCNQ1OT1, SNRPN, PEG3, GNAS</i> (AS1 and A/B ICRs)	<i>H19</i>	NLRP7	73, 74, 77, 78
Oligospermia/azoospermia ^a	<i>PLAGL1/HYMAI, MEST, KCNQ1OT1, SNRPN, PEG3</i>	<i>H19, DLK1-MEG3</i> (IG-DMR)		79–86
Superovulated oocytes	<i>MEST</i> Mouse <i>Peg3, Snrpn, Kcnq1ot1</i>	<i>H19</i> Mouse <i>H19</i>		89, 90
iPS cells		<i>H19</i> Mouse <i>Dlk1-Meg3</i> (IG-DMR)		100, 101

Abbreviations: AS, Angelman syndrome; BiCHM, biparental complete hydatidiform mole; BWS, Beckwith-Wiedemann syndrome; ICR, imprint control regions; iPS, induced pluripotent stem cells; PHP-Ib, pseudohypoparathyroidism type Ib; PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome; TNDM1, transient neonatal diabetes mellitus type 1.

^aICRs involved may differ between cases and for many loss of methylation is partial.

Mutations in *NLRP7*, which is homologous to *NLRP2*, are found in familial recurrent biparental complete hydatidiform mole.^{73–75} A complete hydatidiform mole is usually the product of diploid androgenesis, which results from fertilization of an ‘empty’ egg with subsequent diploidization of the paternal genome, and characterized by hyperproliferative vesicular trophoblast and no fetal development.⁷⁶ Women with biparental complete hydatidiform mole have recurrent complete hydatidiform moles with normal diploid karyotype (one paternal and one maternal genome) but with loss of methylation imprints at multiple ICRs (Table 1).^{74,77,78} *NLRP7* protein is possibly required for both establishment and maintenance of methylation imprints at multiple maternally methylated ICRs.⁷⁴ Interestingly, *NLRP7* is found only in a few species of mammals (human, primate and cow) and there is no strict homolog in rodents, although there are families of related proteins in all mammals.

In sperm from infertile patients with deficiencies such as oligospermia or azoospermia, aberrant methylation imprints at ICRs have been reported (Table 1).^{79–86} Abnormal methylation was observed in not only paternally methylated ICRs but also maternally methylated ICRs. At present, it is unclear how defects in methylation imprints are brought about in these patients, but one explanation could be amino acid sequence variations in DNMT3L.⁸⁷ A rare case of DNMT3L polymorphism was found to be associated with subtelomeric hypomethylation,⁸⁸ but the relevance of such polymorphisms to abnormal

ICR methylation in oligospermic or azoospermic patients remains uncertain.

Although further large studies are needed to establish how common methylation defects are in oligospermic or azoospermic patients, the use of such sperm for ART may increase the risk of imprinting disorders in ART babies. Other reports suggest that proper acquisition of methylation can be perturbed by superovulation; loss of methylation was detected in oocytes at several maternally methylated ICRs (human *MEST*, mouse *Peg3*, *Snrpn* and *Kcnq1ot1* ICRs) and gain of methylation was detected at a paternally methylated ICR (human and mouse *H19* ICRs) (Table 1).^{89,90} A number of studies report that BWS and AS cases are overrepresented in children born through ART.^{42,91–95} However, other studies did not find evidence for increased risk for these diseases,^{96,97} and the relationship between ART and imprinting disorders remains to be established.^{98,99}

Recently, it was reported that the expression state of the imprinted *Dlk1-Dio3* cluster on mouse chromosome 12 is often altered in iPS cells.¹⁰⁰ In the affected mouse iPS cell clones, the ICR within the cluster was abnormally methylated and associated imprinted genes, such as *Meg3*, were abnormally silenced (Table 1). Thus, the unmethylated state of the maternally derived ICR was not maintained during reprogramming. Furthermore, these iPS cell clones contributed poorly to chimeras and failed to support the development of entirely iPS cell-derived mice, whereas embryos derived from iPS cell clones with normal *Meg3* expression developed well.¹⁰⁰ Clone- and gene-specific

aberrations of imprinting were also reported in human iPS cells.¹⁰¹ These studies underscore the importance of the imprint maintenance mechanism in cell reprogramming.

CONCLUSIONS

In this review, we have summarized the current knowledge on the mechanisms of imprint establishment and maintenance and on the human disorders that may have resulted from defects in such mechanisms. Molecular studies in mice have highlighted the importance not only of DNA methylation but also other epigenetic marks and factors, and therefore future studies on human cases should include histone modifications and other factors. Recent technological advances in genome-wide epigenetic analysis will greatly enhance such studies. We also note that the precise mechanism of imprint establishment may differ between ICRs and, furthermore, there are species differences in factors involved: for example, NLRP7 has not been found in mice, and DNMT3L has not been detected in human oocytes.^{102,103} These findings reveal some limitations of mouse studies, despite the great advances made in this tractable model, and underscore the need for additional model systems appropriate for human studies. In this regard, the use of primate species or refinement of the recently developed *in vitro* germ-cell production system¹⁰⁴ will be important for future studies.

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