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Gamete imprinting: setting epigenetic patterns for the next generation

Jacquetta M. Trasler

McGill University-Montreal Children's Hospital Research Institute and the Departments of Pediatrics, Human Genetics and Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada.
Email: jacquetta.trasler@mcgill.ca

Abstract. The acquisition of genomic DNA methylation patterns, including those important for development, begins in the germ line. In particular, imprinted genes are differentially marked in the developing male and female germ cells to ensure parent-of-origin-specific expression in the offspring. Abnormalities in imprints are associated with perturbations in growth, placental function, neurobehavioural processes and carcinogenesis. Based, for the most part, on data from the well-characterised mouse model, the present review will describe recent studies on the timing and mechanisms underlying the acquisition and maintenance of DNA methylation patterns in gametes and early embryos, as well as the consequences of altering these patterns.

Extra keywords: assisted reproductive technologies, DNA methylation, embryogenesis, genomic imprinting, germ cells, human, mouse, oogenesis, spermatogenesis.

Introduction

The term 'epigenetics' encompasses heritable mechanisms of modulating gene expression that do not alter DNA sequence. Three main types of mechanisms, including DNA methylation, RNA-associated silencing and histone modifications (e.g. acetylation and methylation), have been associated with the epigenetic silencing of genes (Egger *et al*. 2004). To date, DNA methylation is the most well-characterised epigenetic modulator that has been shown to have essential functions in the germ line and embryo, as well as in genomic imprinting (variation in the expression of a subset of genes according to their maternal or paternal origin). In particular, genomic imprinting accounts for the requirement of both the maternal and paternal genomes in normal development and, to date, involves approximately 80 genes (http://www.mgu.har.mrc.ac.uk/research/imprinting and linked sites (accessed 17 October 2005)), although expression profiling studies suggest that a much larger number of genes in the genome are imprinted based on evidence of differences in expression between the maternal and paternal alleles (Nikaido *et al*. 2003). Genomic imprinting plays important roles in growth of the embryo, placental function and neurobehavioural processes. Many imprinted genes are characterised by distinct regions called differentially methylated regions (DMRs), where DNA methylation differs between the maternal and paternal alleles. In humans, abnormalities in imprinted genes are associated with cancer, as well as diseases showing growth and neurodevelopmental abnormalities, such as Prader–Willi syndrome, Angelmann syndrome and Beckwith–Wiedemann syndrome (Tycko and Morison 2002).

Deoxyribonucleic acid methylation takes place at the 5-position of cytosine residues within CpG dinucleotides at approximately 30 million sites throughout the mammalian genome, with approximately 60–80% of CpG-containing cytosines being methylated. Erasure, acquisition and maintenance of DNA methylation patterns occur during development. Demethylation can occur passively when methylation is not maintained following DNA replication or it can be carried out actively, although the *in vivo* mechanism or enzyme has not been definitively identified to date. *De novo* methylation allows for the acquisition of new patterns of methylation in the genome, whereas maintenance methylation is required to ensure the propagation of DNA methylation patterns following DNA replication. Methylation of DNA is catalysed by a family of DNA (cytosine-5)-methyltransferases (DNMTs) and is linked to gene expression in that methylation of CpG sites within promoter regions of genes invariably silences transcription. Mammalian DNMTs have been classified according to similarities in their C-terminal domains (Goll and Bestor 2005). The first DNMT to be identified was DNMT1 and it is likely the most important for the maintenance of DNA methylation patterns in the mammalian genome (Bestor *et al*. 1988). More recently four related enzymes, namely DNMT2 (Yoder and Bestor 1998), DNMT3a, DNMT3b (Okano *et al*. 1998) and DNMT3L (Aapola *et al*. 2001; Bourc'his *et al*. 2001), have been characterised. Of the DNMTs, only DNMT1, DNMT3a and DNMT3b are thought to methylate DNA *in vivo*; DNMT3L works in concert with the two other DNMT3 enzymes (Chedin *et al*. 2002; Hata *et al*. 2002; Goll and Bestor 2005).

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Studies over the past 5 years have uncovered when and how DNA methylation patterns are initiated in the germ line and the importance of maintaining the patterns in early embryos. Although most studies have concentrated on the mouse model, as will be reviewed here, important tools have been developed so that conservation in other species can now be examined.

DNA methylation dynamics in the germ line and embryo

As will be outlined in more detail below, marked changes in methylation occur during gametogenesis and embryogenesis. Deoxyribonucleic acid methylation patterns on repeat, single-copy and imprinted sequences are, for the most part, erased in primordial germ cells (PGCs) and then re-acquired at gender-specific times during spermatogenesis and oogenesis. A second period of erasure occurs in the preimplantation embryo, when methylation across much of the genome is lost, with the exception of imprinted genes and some repeat sequences. It is postulated that imprinted gene methylation patterns must be maintained during preimplantation development because it is only in the germ line (male or female, depending on the gene) that imprinted genes acquire the allele-specific methylation that will subsequently be responsible for monoallelic expression in the postimplantation embryo and adult. Because most methylation patterns will be erased in the early embryo, genes other than repeat or imprinted sequences may get methylated in the germ line to play a role in germ cell development, gene expression or chromatin structure during gametogenesis (MacLean and Wilkinson 2005). The genome is remethylated over approximately 2 days in the mouse following the blastocyst stage, leading to methylation patterns that will then, for the most part, persist throughout life. Here, the emphasis will be on imprinted genes as clear examples of genes in which abnormalities in the acquisition of methylation patterns in the germ line or their maintenance in the early embryo impinge markedly on normal development.

Erasure of methylation patterns in the germ line

Evidence to date indicates that PGCs carry parental methylation profiles as they migrate towards the genital ridge. As they enter the genital ridge, at approximately 10.5 days of gestation in the mouse, they undergo genome-wide demethylation. Early studies using methylation-sensitive restriction enzymes and Southern blot or polymerase chain reaction showed that the genomes of PGCs appeared to become demethylated by 13.5 days of gestation (Monk *et al*. 1987; Chaillet *et al*. 1991; Kafri *et al*. 1992; Brandeis *et al*. 1993). More recent studies have used bisulfite sequencing, a sensitive technique that allows detection of all methylated cytosines in a gene of interest in small numbers of cells, and, if needed, in an allele-specific manner (Clark *et al*. 1994). Bisulfite sequencing showed that imprinted genes, including *Peg3*, *Kcnq1ot1* (also known as *Lit 1*), *Snrpn*, *H19*, *Rasgrf1* and *Gtl2*, as well as non-imprinted genes, such as *α-actin*, become demethylated between 10.5 and 13.5 days of gestation (Hajkova *et al*. 2002; Li *et al*. 2004). Certain sequences, such as the repetitive elements intracisternal A particle (IAP), long interspersed nuclear element 1 (LINE1) and minor satellites, may be treated differently because they appear to be only partially demethylated in PGCs (Hajkova *et al.* 2002; Szabo *et al*. 2002; Lane *et al*. 2003; Lees-Murdock *et al*. 2003).

Alternative approaches, such as monitoring the expression of imprinted genes, have also been used to assess epigenetic reprogramming events in PGCs. Prior to the erasure of epigenetic marks, imprinted genes would be expected to be monoallelically expressed, whereas the appearance of biallelic expression would be an indication of erasure of marks. This approach was used by Szabo *et al.* (2002), who showed that although four imprinted genes, including *H19* and *Snrpn*, were monoallelically expressed at 9.5 days of gestation, *Snrpn* was biallelically expressed by day 10.5, as were the other genes by day 11.5. Thus, the timing was similar to that noted in the methylation studies described above. Other investigators have examined PGC reprogramming by analysing embryos derived by somatic cell nuclear transfer using PGC nuclei at different stages of development; these experiments also provide evidence that methylation imprints are erased between 10.5 and 12.5 days of gestation (Lee *et al*. 2002; Yamazaki *et al*. 2003).

Thus, it would appear from several different types of studies performed in mice, that erasure of methylation patterns occurs in male and female germ cells between approximately 10.5 and 12.5 days of gestation. The mechanisms underlying the genomic demethylation in PGCs are unclear at present, but have been suggested to involve an active process (Hajkova *et al*. 2002). The timing raises several issues. It will be important to confirm whether a similar reprogramming event occurs in other species and whether alterations in demethylation due to *in utero* events, such as dietary deficiency or drugs, can have long-lasting effects in the offspring. Intriguingly, a few studies have suggested that epigenetic abnormalities in germ cells may not be erased in PGCs. For instance, in one study in the mouse, the abnormal methylation status of an IAP repeat element within the *Agouti* locus was found to be heritable (Morgan *et al*. 1999). In another study, exposure of rat fetal germ cells to vinclozolin resulted in methylation abnormalities that were passed through several generations (Anway *et al*. 2005).

Developmental timing and mechanisms underlying the acquisition of DNA methylation in germ cells

Timing

Deoxyribonucleic acid methylation patterns are acquired at different developmental times in the male and female germ

lines. In the male, DNA methylation patterns begin to be acquired before birth in gonocytes and are complete for most sequences after birth, before the pachytene phase of meiosis (Kafri *et al*. 1992; Walsh *et al*. 1998; Davis *et al*. 1999, 2000; Ueda *et al*. 2000; Lees-Murdock *et al*. 2003; Li *et al*. 2004). In the female germ line, gametic methylation is acquired postnatally, following pachytene (Chaillet *et al*. 1991; Ueda *et al*. 1992; Brandeis *et al*. 1993; Stoger *et al*. 1993; Kono *et al*. 1996; Walsh *et al*. 1998; Lucifero *et al*. 2002, 2004*a*; Obata and Kono 2002).

In the male germ line, increases in DNA methylation, as measured by staining with an antibody to methylated cytosine, are first seen by immunocytochemistry in gonocytes between 15.5 and 18.5 days of gestation (Coffigny *et al*. 1999). Methylation analysis of individual genes yields similar results. In germ cells, the maternally expressed, paternally methylated imprinted gene *H19* first acquires methylation between 15.5 and 18.5 days of gestation; *H19* methylation continues to increase in postnatal germ cells and is complete by pachytene, persisting in spermatozoa (Davis *et al*. 1999, 2000; Ueda *et al*. 2000). Interestingly, the paternal alleles of *H19* acquire their methylation before the maternal alleles, providing evidence that paternal alleles may have a 'memory' of their origin (Davis *et al*. 1999, 2000). In addition to *H19*, the imprinted genes *Gtl2* (Takada *et al*. 2002) and *Rasgrf1* (Yoon *et al*. 2002) are also methylated on the paternal allele. The DMRs of these genes first begin to acquire methylation between 12.5 and 17.5 days of gestation; as for *H19*, methylation of *Gtl2* and *Rasgrf1* continues during postnatal spermatogenesis (Li *et al*. 2004). Other than imprinted genes, repetitive DNA elements, such as LINE1, IAP and minor satellite sequences, also acquire methylation in prenatal gonocytes (Walsh *et al*. 1998; Lees-Murdock *et al*. 2003). Together, the methylation studies indicate that the methylation of imprinted genes is complete before meiosis. Further, functional support that male germ cells have acquired their methylation patterns on imprinted genes before the haploid phase of spermatogenesis comes from intracytoplasmic round spermatid and sperm injections, where the expression of imprinted genes in the resulting embryos was equivalent in round spermatid- and sperm-derived offspring (Shamanski *et al*. 1999).

In the female germ line, DNA methylation studies on imprinted genes and some repeat sequences have shown the postnatal oocyte growth phase to be the time when methylation patterns are first acquired. In early studies, examination of a few CpG sites in the imprinted gene *Igf2r* (Brandeis *et al*. 1993; Stoger *et al*. 1993; Kono *et al*. 1996), the *RSVIgmyc* and *MPA434* transgenes (Chaillet *et al*. 1991; Ueda *et al*. 1992) and the IAP retroviral sequence (Walsh *et al*. 1998) first suggested that *de novo* methylation occurs during the maturation of non-growing oocytes to metaphase (M) II oocytes. Support for the functional importance of the methylation of imprinted genes during oocyte growth comes from elegant nuclear transplantation experiments. Kono *et al.* (1996) first showed that parthenogenetic embryos containing one genome from a non-growing oocyte from a neonatal mouse and the other genome from a fully grown oocyte developed for 3 days longer than normal parthenogenotes. The findings suggested that oocyte genomes at different stages during oocyte growth are functionally different. Further nuclear transplantation studies on oocytes at different stages of growth, together with the examination of imprinted gene expression in the resulting embryos, provided evidence that imprint acquisition occurs during the postnatal oocyte growth phase, along with the suggestion that some genes gain their imprints earlier than others (Bao *et al*. 2000; Obata and Kono 2002). Complementary bisulfite sequencing experiments have been used to directly characterise the methylation of four paternally expressed genes in oocytes at different stages of oocyte growth (Lucifero *et al*. 2002, 2004*a*). Methylation imprint acquisition occurred in a gene-specific manner, with *Snrpn* acquiring methylation first, followed by *Igf2r* and *Peg3*, whereas *Peg1* only acquired methylation at late stages of oocyte growth. For *Snrpn*, the acquisition of methylation was closely related to oocyte diameter such that *Snrpn* was unmethylated in oocytes with a diameter of 20–50 μm and largely methylated in oocytes with a diameter of 60–80 μm (Lucifero *et al*. 2004*a*). Seemingly analagous to the case for *H19* 'memory' described above, the maternal alleles of *Snrpn* became methylated at an earlier time in oocyte growth than the paternal alleles, providing further evidence that the parental alleles may not be equivalent following erasure of epigenetic marks in PGCs.

Mechanisms

In somatic tissues, DNMT1 is predominantly involved in the maintenance of DNA methylation patterns following replication, whereas the DNMT3 enzymes have been implicated in the acquisition of new methylation patterns or *de novo* methylation. In the male, DNMT1 is not expressed in Day 15.5–18.5 prenatal gonocytes at the time when methylation patterns are initially acquired (Sakai *et al*. 2001; La Salle *et al*. 2004). However, DNMT3a and DNMT3L are expressed at the RNA and/or protein levels in prenatal germ cells (La Salle *et al*. 2004; Sakai *et al*. 2004). In addition, gene-targeting experiments have identified DNMT3a and DNMT3L as the predominant enzymes involved in the initial methylation of repetitive and imprinted sequences in the male germ line (Bourc'his and Bestor 2004; Kaneda *et al*. 2004; Webster *et al*. 2005). The absence in prenatal male germ cells of either DNMT3a or DNMT3L results in the failure of spermatogenesis and infertility. In addition, DNMT1, DNMT3a, DNMT3b and DNMT3L are expressed and developmentally regulated in the postnatal testis (Jue *et al*. 1995; Mertineit *et al*. 1998; La Salle *et al*. 2004). The precise roles of each enzyme in the maintenance and *de novo* methylation during postnatal spermatogenesis have not yet been delineated and will

likely require enzyme- and germ cell-specific gene-targeting or ablation experiments.

With respect to enzymes involved in DNA methylation in the female germ line, we described an oocyte (DNMT1o)-specific form of DNMT1 (Mertineit *et al*. 1998). It was shown that DNMT1o was the only form of DNMT1 expressed in oocytes and preimplantation mouse embryos (Ratnam *et al*. 2002) and it was initially postulated to play an important role in the acquisition of methylation patterns in oocytes. However, when *Dnmt1o* was subsequently knocked out in a mouse model, examination of gene-specific DNA methylation, as well as nuclear transplantation experiments, definitively showed that DNMT1o is not required for the establishment of methylation patterns in oocytes (Howell *et al*. 2001). It has been reported that *Dnmt3a*, *Dnmt3b* and *Dnmt3L* are all expressed in oocytes at the time when methylation imprints are acquired (Lucifero *et al*. 2004*a*). The DNMT3a and DNMT3L enzymes are key enzymes for the acquisition of methylation patterns in oocytes because the absence of either enzyme in the female germ line by gene-targeting results in the lack of establishment of methylation imprints (Bourc'his *et al*. 2001; Kaneda *et al*. 2004).

Maintenance and propagation of epigenetic information in the early embryo

Timing

Following fertilisation, DNA methylation decreases markedly as the embryo develops to the blastocyst stage; the demethylation process occurs across much of the genome, with the exception of imprinted genes and some repetitive sequence elements. Interestingly, in the mouse, the male pronucleus DNA becomes demethylated within approximately 4 h of fertilisation (Mayer *et al*. 2000; Oswald *et al*. 2000; Santos *et al*. 2002), indicating an active process, whereas the DNA of the female pronucleus is demethylated more slowly throughout preimplantation development, most likely via passive demethylation, where methylation is not maintained by DNMTs following replication (Howlett and Reik 1991; Rougier *et al*. 1998). Methylation is postulated to be preserved at imprinted loci to allow for gender-specific monoallelic expression of genes required for normal development (Olek and Walter 1997; Tremblay *et al*. 1997; Hanel and Wevrick 2001; Reik and Walter 2001). Genome-wide *de novo* methylation occurs at the end of preimplantation development and coincides with differentiation events taking place after the fifth cell cycle (Santos and Dean 2004).

Mechanisms

The rapid loss of DNA methylation from the paternal genome indicates an active process, although the enzyme(s) involved has not been identified. To date, one enzyme, namely DNMT1o, has been implicated in the maintenance of methylation on imprinted genes and repeat sequences during preimplantation development. Although, as indicated above, the oocytes of DNMT1o-deficient mothers showed normal levels of DNA methylation, all offspring of DNMT1o-deficient mothers died during late gestation and showed methylation and expression abnormalities restricted to imprinted genes (Howell *et al*. 2001). These results indicate that DNMT1o is essential for maintenance methylation during preimplantation development. However, immunocytochemistry experiments have shown DNMT1o to be present in the nucleus only at the eight-cell stage, whereas it is in the cytoplasm at all other stages of preimplantation development in the mouse; it is thus unclear what enzyme/activity maintains DNA methylation at stages other than the eight-cell stage.

Epigenetics, genomic imprinting and the germ line: implications for disease, assisted reproductive technologies and stem cell research

Human conditions associated with abnormalities in DNA methylation include growth and genomic imprinting disorders, molar pregnancies and childhood cancer. Several recent studies have linked the genomic imprinting disorders Beckwith–Wiedemann syndrome (DeBaun *et al*. 2003; Gicquel *et al*. 2003; Maher *et al*. 2003; Halliday *et al*. 2004) and Angelman syndrome (Cox *et al*. 2002; Orstavik *et al*. 2003) with the use of assisted reproductive technologies (Lucifero *et al*. 2004*b*). In the case of the genomic imprinting disorders, the absence of maternal methylation imprints was found, involving the *KCNQT1OT1* locus in Beckwith–Wiedemann syndrome and the *SNRPN* locus in Angelman syndrome. However, it was unclear whether the birth defects were related to the underlying infertility diagnosis that led the couples to use assisted reproductive technologies or to the techniques being used. Assisted reproductive technologies are of concern because they include superovulation and the manipulation of gametes and early embryos at times when genomic imprints may be susceptible to perturbations. In particular, in several different animal studies, embryo culture conditions were associated with abnormalities in imprinting or the growth/outcome in the offspring (Doherty *et al*. 2000; Khosla *et al*. 2001; Young *et al*. 2001). However, the fact that no specific human assisted reproduction technique could be linked to the imprinting disorders provided support for the suggestion that epigenetic factors underlying female or male infertility may also be involved. Two studies provide evidence for a role of epigenetic abnormalities in infertility. In one, the methylation of the imprinted genes *MEST* and *H19* was examined in the sperm from normal fertile males compared with oligospermic males (Marques *et al*. 2004). Although *MEST*, which is normally unmethylated in sperm, was unaffected, *H19* showed lower levels of methylation in the sperm DNA of the oligospermic men. The second

study reported an increased incidence of imprinting defects in patients with Angelman syndrome born to subfertile couples with suggestive evidence of an interaction between subfertility and assisted reproductive technologies (Ludwig *et al*. 2005). More research is needed in both human and animal models to examine possible epigenetic causes for infertility. Furthermore, modelling specific techniques used in human assisted reproductive technologies in other species will help identify those procedures most likely to perturb imprints.

Human hydatidiform moles typically result when there is only paternal (or androgenetic) contribution to a pregnancy, are composed of hyperplastic placental tissue and cause gestational trophoblastic disease. An inherited form of hydatidiform moles, indistinguishable from the typical androgenetic moles but carrying both maternal and paternal genomes (i.e. biparental, as in normal gestations), has been described (Helwani *et al*. 1999; Moglabey *et al*. 1999; Judson *et al*. 2002; El-Maarri *et al*. 2003). However, the maternal genomes of the inherited biparental moles appear to lack methylation imprints (and, thus, look and act more like paternal genomes or have a paternal 'epigenotype') as a result of a postulated defect in the female germ line (Judson *et al*. 2002). The defect in the female germ line is not likely to be due to a defect in one of the DNMTs because no mutations were detected (Hayward *et al*. 2003). It is possible that an as yet unidentified factor, such as a protein or an enzyme, involved in regulating the establishment of imprints is affected; identifying the factor may shed new light on imprint establishment mechanisms in humans, as well as other species.

The proper maintenance and propagation of DNA methylation information in early embryos have important implications for stem cell research (Allegrucci *et al*. 2004). Embryonic stem (ES) cells derived from the inner cell mass of human blastocysts can be cultured *in vitro*, are pluripotent and capable of differentiating into different types of cells and have been proposed for use in regenerative medicine. However, there is concern that epigenetic abnormalities present in human embryos may be propagated in any ES cell lines derived from them. Several animal studies have raised concerns about the stability of epigenetic marks in ES cells (Dean *et al*. 1998; Humpherys *et al*. 2001). Recent studies on human ES cells have reached different conclusions. In one study, the allele-specific expression of six imprinted genes, as well as the methylation profiles of three imprinting control regions, were examined in human ES cell lines (Rugg-Gunn *et al*. 2005). Although a relatively small number of imprinted genes were examined, the authors reported, for the most part, monoallelic expression of the imprinted genes and normal methylation. In the second study, Maitra *et al.* (2005) found clear evidence of both genomic and epigenetic alterations in late-passage human ES cell lines. These human ES cell studies indicate the need for further research and suggest the importance of developing sensitive, genome-wide screening methods to detect epigenetic and genetic abnormalities in any human ES lines and their derivatives that are destined for therapeutic purposes.

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