

# Genomic imprinting and reproduction

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## Abstract

**Genomic imprinting is the parent-of-origin specific gene expression which is a vital mechanism through both development and adult life. One of the key elements of the imprinting mechanism is DNA methylation, controlled by DNA methyltransferase enzymes. Germ cells undergo reprogramming to ensure that sex-specific genomic imprinting is initiated, thus allowing normal embryo development to progress after fertilisation. In some cases, errors in genomic imprinting are embryo lethal while in others they lead to developmental disorders and disease. Recent studies have suggested a link between the use of assisted reproductive techniques and an increase in normally rare imprinting disorders. A greater understanding of the mechanisms of genomic imprinting and the factors that influence them are important in assessing the safety of these techniques.**

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## What is genomic imprinting?

Genomic imprinting is the parent-of-origin specific gene expression and is determined by epigenetic modification of genes, such that gene transcription is altered while the actual gene sequence remains unchanged. Genomic imprinting results in only one inherited copy of the relevant imprinted gene being expressed in an embryo. For paternally imprinted genes, the paternal allele is epigenetically modified to prevent transcription, ensuring that the embryo has only mono-allelic expression from the maternally inherited copy. The opposite is true of maternally imprinted genes, when only the copy inherited from the father is expressed. The fact that particular genes are differentially expressed, according to their parent-of-origin, means that during development the parental genomes are functionally non-equivalent (Surani 1998). Genomic imprinting is vital for normal gene expression patterns in an individual, with errors sometimes resulting in inappropriate gene transcription or repression. Within the mouse genome, approximately 80 imprinted genes have so far been identified (Beechey *et al.* 2005). It is likely that there will be a similar number of imprinted genes in humans, although fewer have been found to date. Except where stated, this review refers to work on the mouse, as this species has been by far the most extensively studied species.

Within the mammalian genome, the majority of cytosine residues found as CpG dinucleotides (i.e. those cytosines positioned next to a guanine residue) have a methyl group added to their carbon 5 position (Costello & Plass 2001). It is this addition of the methyl group, referred to as

DNA methylation, that is proposed as the key mechanism (certainly the best studied mechanism) regulating imprinting. It is important to point out, however, that the majority of methylated DNA in the genome is not concerned with genomic imprinting. Heavy methylation of DNA results in a more condensed structure which is resistant to transcription. Thus, if an animal inherits a methylated copy of a gene from its mother and a non-methylated copy from its father, the maternal copy will have its transcription repressed leaving the paternal copy as the only active gene. DNA methylation is an epigenetic modification which can be inherited in a stable manner but is also reversible, allowing gender specific patterns to be initiated in germ cells.

## Why did genomic imprinting evolve?

The most commonly proposed theory explaining the development of genomic imprinting is the genetic conflict or parental investment theory (Moore & Haig 1991). This theory arose from the observation that many imprinted genes are implicated in the growth and development of the mammalian fetus or placenta. In addition to this, imprinted genes have been shown to exhibit directionality in their actions; that is the majority of the paternally expressed genes, such as *Igf2* and *Peg3*, promote fetal growth and nutrient uptake while in contrast the maternally expressed genes, such as *Igf2R* and *Gnas*, tend to curb fetal growth (Reik & Dean 2001, Tycko & Morison 2002). Females who could restrict fetal growth and

produce more offspring from their limited resources would, in the long term, be more successful. In contrast, males would derive long-term benefit from their progeny being larger and stronger even if they achieved this to the detriment of the mother by utilising more maternal nutrients.

An alternative theory to account for the presence of imprinted genes within the genome is the evolvability model (Beaudet & Jiang 2002). This predicts that species which have genomic imprinting are more able to evolve in response to environmental pressures, as they can induce rapid changes by altering which of the two alleles is silenced and which is expressed. An individual organism can carry an allele which promotes growth that, while imprinted, has no phenotypic effects. Thus, if increased growth becomes advantageous, the relevant allele is already present in the gene pool: by rapid reversal of the imprinting, the allele can be expressed. There is also the 'ovarian time bomb' concept which proposes that genomic imprinting evolved to protect the female from ovarian disease: Varmuza & Mann (1994) hypothesised that imprinting could limit the level of growth and development of any parthenogenetic embryos within the ovary, thereby preventing malignant trophoblast formation.

### How are DNA methylation patterns regulated?

DNA methyltransferases (Dnmts) carry out methylation of DNA; these can be broadly divided into Dnmt1, Dnmt2 and Dnmt3 families. The three families of Dnmts are related, albeit distantly, and are believed to have diverged from their common ancestors prior to the separation of the animal and plant kingdoms (Howell *et al.* 2001). Members of both the Dnmt1 and Dnmt3 families have been identified as having active transmethylase activity and their functions have been partially elucidated, with the Dnmt3 family being primarily concerned with laying down new methylation patterns while the Dnmt1 family appears to be mainly involved in the maintenance of these patterns during cell division. Initial studies on Dnmt2 did not find any active methylation function for this protein but more recent research has challenged this concept, with work by several groups finding that this enzyme can act as a methyltransferase which targets a very specific DNA sequence, explaining the low level of identifiable activity (Liu *et al.* 2003, Hermann *et al.* 2003, Tang *et al.* 2003). Although capable of binding to methylated DNA, the definitive binding specificity of Dnmt2 has yet to be determined (Hermann *et al.* 2003). Golding & Westhusin (2003) have shown that Dnmt2 is actually the most prevalent Dnmt in the bovine adult ovary and testis.

### Laying down of methylation patterns

To allow reprogramming of the germ cell, the genome must undergo demethylation (as described later). Once the initial imprints have been removed the appropriate

new pattern must be established, thus ensuring that the paternal- and maternal-specific imprints are laid down in the sperm and oocyte respectively. The enzymes which are capable of laying down the new methyl groups onto previously unmethylated DNA are from the Dnmt3 family. Members of this family which have active transmethylase activity are Dnmt3a and Dnmt3b, which share a high degree of sequence homology but have been shown to have different expression patterns and timing through development. The third member of this family, Dnmt3l, shares sequence homology with the other enzymes but is missing the catalytic domain needed to add methyl groups onto DNA. After both examining the localisation of this protein and using mice with a disrupted *Dnmt3l* gene, a role in the establishment of maternal imprints in the oocyte has been hypothesised for this enzyme, as discussed below.

### Maintenance of methylation

When a methyl group is added onto already hemimethylated DNA during cell replication (necessary if the daughter cells are to maintain the methylation pattern of the cell undergoing mitosis), the process is termed maintenance methylation. Dnmt1 has the primary responsibility for maintaining the methylation status of DNA. The most common form of this methyltransferase is that found in all somatic cells, Dnmt1s, and has been shown to be vital for development. In addition, there are two splice variants identified which are specific to the germ cells and early embryo. Dnmt1p is found in pachytene spermatocytes whilst Dnmt1o is only identifiable in the oocyte and pre-implantation embryo. It is not until embryonic day 7 (E7) that the embryo is capable of producing full-length Dnmt1s protein.

Interestingly, although Dnmt1 has been identified as the main maintenance methylase *in vivo*, studies *in vitro* have shown that this enzyme has a higher *de novo* methylase activity than either Dnmt3a or Dnmt3b. *In vivo*, Dnmt1 *de novo* methylase activity has yet to be found, but the possible implications of this *in vitro* activity should be borne in mind (Howell *et al.* 2001).

### How does methylation lead to repressed gene transcription?

There are two main mechanisms by which the methylation of DNA can prevent the transcription of genes. The first of these is by the methyl group causing direct interference preventing particular transcription factors from binding to methylated DNA (Iguchi-Arigo & Schaffner 1989). The second mechanism results from methyl-binding domain proteins (MBDs) binding to methylated DNA.

Of the MBDs identified to date, MBD1 to MBD3 and methyl CpG-binding protein 2 (MeCP2) are involved in transcriptional repression (Nan *et al.* 1997, Fujita *et al.* 1999, Ng *et al.* 1999), while MBD4 is thought to have a role as a mismatch repair protein (Hendrich *et al.* 1999).

MBD1 and MeCP2 both contain transcriptional repression domains which act via histone deacetylases (HDACs). HDACs cause local deacetylation of the histone tails which, in turn, results in remodelling of the chromatin into a more condensed structure that is resistant to transcription (Taunton *et al.* 1996). MBD1 mediates transcriptional repression through recruitment of a histone methylase capable of binding HDACs (Ng *et al.* 2000, Fujita *et al.* 2003), while MeCP2 acts to bind a co-repressor complex containing an HDAC (Jones *et al.* 1998, Nan *et al.* 1998), although MeCP2 has also been shown to cause transcription repression in the absence of HDAC activity (Nan *et al.* 1998, Yu *et al.* 2000). MBD2 and MBD3 are both components of a large protein complex, MeCP1 (Feng & Zhang 2001). MeCP1 binds methylated DNA in a non-sequence-specific manner. The binding of MeCP1 to methylated DNA is due to the presence of MBD2 in the complex (Ng *et al.* 1999). Interestingly, the mammalian form of MBD3 appears not to bind directly to methylated DNA (Hendrich & Bird 1998). The MeCP1 complex binds methylated DNA less tightly than MeCP2, which suggests that long-term transcriptional repression may be maintained by the permanent binding of MeCP2, with more transient transcriptional silencing determined by the binding of the MeCP1 complex (Ng *et al.* 1999). In addition to the MBD family, there is a further binding protein termed Kaiso which is capable of methylation-dependent repression of gene transcription. Although it is not an MBD-containing protein, it is capable of binding to methylated DNA via its zinc finger (Prokhortchouk *et al.* 2001). Kaiso has been shown to be a vital component of amphibian development; blocking translation of this protein is lethal (Ruzof *et al.* 2004) but the extent of its role in mammalian systems has yet to be established. Methylation-dependent transcriptional repression is covered by many good reviews such as Wade (2001) and Li (2002).

Transgenic studies show that mice lacking MBD1 have no observable phenotype, although problems within the nervous system are evident at the molecular level (Zhao *et al.* 2003). *Mbd2* knockout (KO) mice are also viable, although they exhibit impaired maternal behaviour (Hendrich *et al.* 2001). *Mbd3* null mutations are embryo lethal (Hendrich *et al.* 2001). The abnormal phenotype of *Mecp2* KO mice develops from several weeks of age and is lethal by 8 weeks of age, with all known abnormalities having their origin in the nervous system (Guy *et al.* 2001). The fact that *Mbd1*, *Mbd2* and *Mbd3* KO mice have no apparent phenotype outside of the nervous system suggests that there is a degree of redundancy within the MBD-mediated system of transcription control. Although a double KO of *Mbd2* and *Mecp2* has demonstrated that both these proteins function in separate pathways, this does not rule out co-operation between other members of the MBD family (Guy *et al.* 2001).

## Genomic imprinting in germ cells and embryos

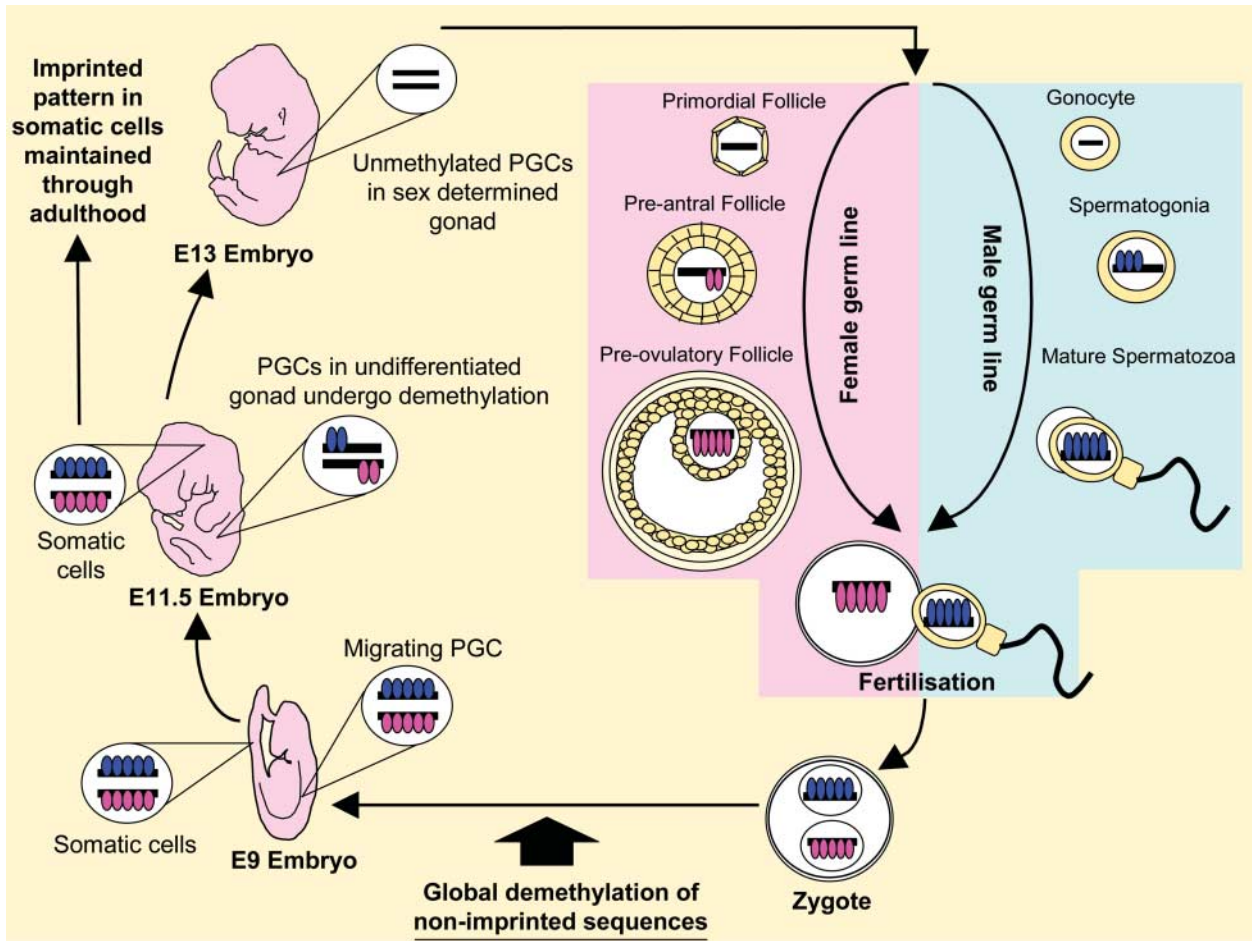
### Primordial germ cells

When primordial germ cells (PGCs) are first seen in the mouse embryo at E7 they, and the surrounding somatic cells, carry the maternally and paternally inherited imprinting patterns. This DNA methylation pattern is maintained in PGCs as they migrate to the developing gonad. Coincident with their arrival in the gonadal ridge, the mouse PGCs begin to undergo global demethylation from around E11.5 to remove their inherited imprinting pattern. During this period, DNA methylation of the somatic cells is maintained (Fig. 1). Demethylation of germ cells is clearly vital if the correct sex-specific epigenetic information is to be subsequently laid down during oocyte and sperm maturation. Demethylation is complete by E13–14, correlating to the period when the male and female mouse PGCs begin to enter mitotic and meiotic arrest respectively. It has been suggested that mitotic/meiotic arrest might necessarily follow demethylation because replication of unmethylated DNA has an increased risk of unrepressed retro-transposons moving and causing mutations (Walsh *et al.* 1998). The time at which this demethylation occurs, and also the amount of methylation lost, appears to be identical regardless of the gender of the embryo (Hajkova *et al.* 2002). Whether the loss of methylation occurs by a passive or active mechanism or a combination of both is not yet known, although the speed with which this occurs would suggest involvement of an active mechanism.

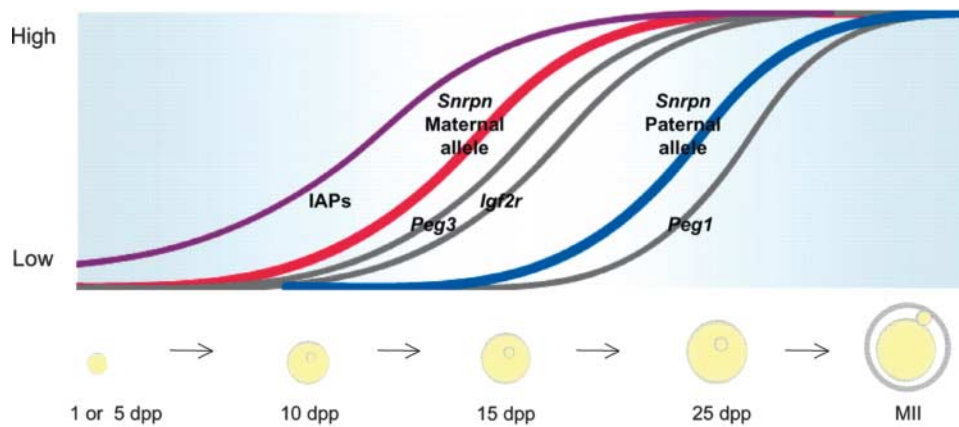
### Oocyte development

In female embryos, the gonad forms as an ovary with germ cells forming primordial follicles. As long as the primordial follicle and the oocyte contained within it are not activated to enter the growing population, the methylation level of the oocyte genome remains low and unchanged. It is during the growth phase of the oocyte that the maternal imprints are laid down on the genome (Fig. 1). The imprints are not all established at the same time; instead, each imprinted gene has a specific time at which it will become methylated (Fig. 2). Obata & Kono (2002) analysed parthenogenetic embryos created by nuclear transfer of oocyte nuclei from different stages of follicle development, with the aim of establishing the timing of the maternal imprinting within the oocytes, and showed that *Snrpn*, *Znf127* and *Ndn* genes are imprinted early in follicle development during the primordial to primary follicle stages, whilst imprinting of *Peg3*, *Igf2r* and *p57<sup>KIP2</sup>* happens at the secondary follicle stage. There are also genes which become imprinted at even later stages of follicle development, including *Peg1/mest* during tertiary to early antral stages and *Impact* which only becomes imprinted in the oocyte within an antral follicle (Obata & Kono 2002). A further study by Lucifero *et al.* (2004)





**Figure 1** The maternal (pink shaded region) and paternal (blue shaded region) imprints are laid down during germ cell development so that by the time the oocyte and sperm are fully mature the correct pattern of DNA methylation is present on the genome (female imprints, pink ovals; male imprints, blue ovals). After fertilisation (yellow-shaded area), both parental genomes undergo global demethylation of non-imprinted sequences: imprinted genes are protected from this process. During early embryo development the imprinted genes of both the somatic and PGC retain the parental imprints. From E11.5 the primordial germ cells begin to undergo demethylation to erase the inherited parental imprints, but the somatic cells of the embryo maintain the parental imprints through embryo development and into adulthood. The process of PGC demethylation is complete by E13. Subsequent reprogramming of the germ cells occurs when the gender-specific imprinting patterns are once more laid down.



**Figure 2** Methylation levels of individual imprinted genes and non-imprinted regions of the genome were assessed over the period of oocyte growth and development. These results demonstrate the gene-specific nature of methylation of the genome, with some genes imprinted early on in follicle development while others are imprinted much later. dpp, days post partum; MII, Metaphase II; IAP, intracisternal A particles; Reproduced from Lucifero *et al.* (2004) with permission from Oxford University Press.

investigated the methylation of imprinted genes by dissecting follicles from ovaries of different postnatal ages and examining the oocytes. This confirmed the earlier Obata & Kono, 2002 study in that *Peg3*, *Igf2r* and *Snrpn* began to gain methylation earlier in development than *Peg1*. By the early antral stages, some differentially methylated regions (DMRs) were fully methylated in all genes other than *Peg1*, while it was not until oocytes were fully mature that *Peg1* appeared to undergo rapid *de novo* methylation. Thus, the imprinting pattern of the oocyte is not fully laid down until it is within a mature follicle ready to ovulate. This has clear implications for assisted reproductive techniques (ARTs), where follicle and oocyte maturation is usually artificially stimulated; any such process must support the correct completion of oocyte imprinting.

Over the period of oocyte growth, the general level of DNA methylation increases as both the appropriate maternal pattern of imprinting is laid down and non-imprinted sequences also become methylated (Fig. 3). The Dnmt involved has yet to be identified, although it has been suggested that one or more members of the Dnmt3 family could be responsible. Dnmt3a, 3b and 3l are all expressed during postnatal oocyte growth. Dnmt3l is expressed at a higher level than either Dnmt3a or Dnmt3b, although all three have maximal expression levels occurring at approximately the same stage of oocyte development (Lucifero *et al.* 2004). Dnmt1s protein is not found in either growing oocytes or in pre-implantation embryos. Instead, an alternatively spliced, more stable transcript, Dnmt1o, is expressed at these stages. In the growing oocyte, Dnmt1o is found in both the cytoplasm and the germinal vesicle (Fig. 4), but once the oocyte is fully matured, it is localised to the cytoplasm where it is stored until it is required during later embryo development (Carlson *et al.* 1992, Mertineit *et al.* 1998). Since *Dnmt1o* translation only occurs early in oocyte development, the stability of this form of Dnmt1 is clearly important.

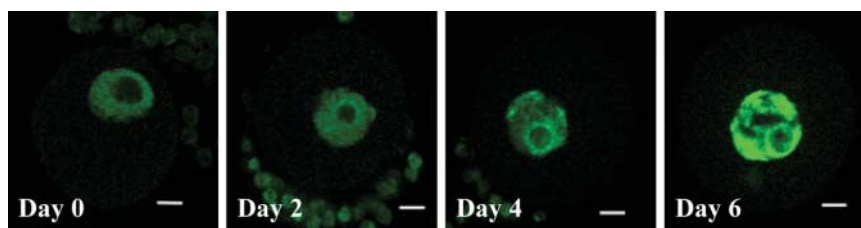
Dnmt3l does not have the active transmethylase activity which is a characteristic of the other Dnmt3 family proteins, Dnmt3a and 3b. However, Hata *et al.* (2002) found that Dnmt3l expression was vital if normal maternal imprints were to be laid down in the oocyte and that this function may be mediated through its ability to bind and

co-localise with both Dnmt3a and 3b. Mice with a disrupted *Dnmt3l* gene are sterile. Males produce no mature sperm (see below); females undergo apparently normal oocyte growth and the resulting oocytes can be fertilised, but the absence of maternal *Dnmt3l* is embryo lethal to heterozygote offspring by E9.5 (Bourc'his *et al.* 2001). Interestingly, a conditional KO with disrupted *Dnmt3a* in the germ cells has an almost identical phenotype to the *Dnmt3l*<sup>-/-</sup> mouse (Kaneda *et al.* 2004): when females whose oocytes lacked *Dnmt3a* were crossed with wild-type males all offspring died by E11.5, with embryos lacking methylation on the normally methylated maternally imprinted genes (resulting in inappropriate gene expression). This study demonstrates the essential role of *Dnmt3a* in the establishment of maternal imprints. Kaneda *et al.* (2004) also investigated the role of *Dnmt3b* using a conditional KO; these animals were found to be phenotypically normal and were able to produce viable offspring.

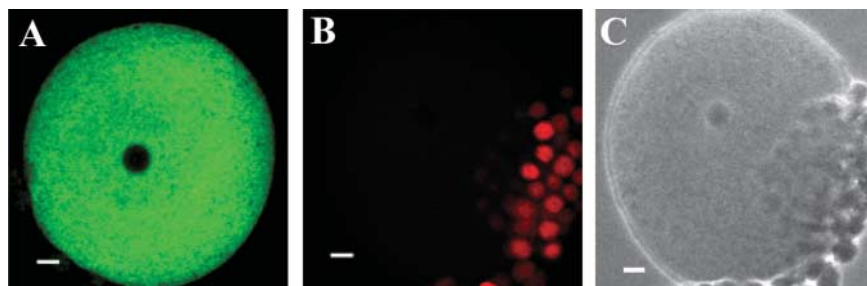
### Sperm development

As with the oocyte, new imprints are laid down as sperm develop (Fig. 1), with the increase in DNA methylation levels not just attributable to the establishment of paternal imprints but also the methylation of other non-imprinted sequences, such as intracisternal A particles (IAPs) becoming methylated (Walsh *et al.* 1998). The paternally expressed (i.e. maternally imprinted) human *MEST/PEG1* gene is demethylated during fetal life and then remains unmethylated through all stages of sperm development in adult life. Ueda *et al.* (2000) analysed the methylation level of an imprinted gene, *H19*, in male germ cells and found that the *H19* imprint is laid down early in germ cell development before meiosis occurs. The same result was found in humans, with the *H19* gene becoming methylated before meiosis at the spermatogonial stage of development (Kerjean *et al.* 2000). In general, though, there is less information about the laying down of imprinting patterns during sperm development compared with what is known about imprinting in oocytes.

The resumption of mitotic division of male germ cells at puberty coincides with an increase in the level of Dnmt1 within the spermatocytes. During the early stages of



**Figure 3** Global methylation level of the oocyte increases over the period of growth and development associated with follicle development from pre-antral stages to full maturity. Confocal images showing oocytes stained with 5-methyl-cytosine antibody (Eurogentec, Seraing, Belgium) and an FITC fluorescent secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Late pre-antral follicles were dissected from 3-week-old mouse ovaries and cultured as in Spears *et al.* (1994), with follicles developing to the Graafian stage over a 6-day period. Follicles were removed from culture and oocytes recovered on days 0, 2, 4 and 6 of culture before being fixed and stained. White scale bars represent 10  $\mu\text{m}$ .



**Figure 4** The oocyte expresses only Dnmt1o (and not Dnmt1s). Until oocyte maturation is complete, Dnmt1o is localised in the cytoplasm and germinal vesicle – but not the nucleolus – of growing oocytes. The figure shows an oocyte from a mid-antral (i.e. not yet fully mature) follicle. (A) Immunocytochemistry using the PATH52 antibody that recognises both Dnmt1s and Dnmt1o (kindly donated by T Bestor, Columbia University). The image shows localisation of Dnmt1 in both the cytoplasm and the germinal vesicle of the oocyte, with only the nucleolus remaining unstained. (B) Immunocytochemistry using the UPT82 antibody which detects only Dnmt1s (kindly donated by J R Chaillot, University of Pittsburgh). The cumulus cells are heavily stained while, as expected, the oocyte remains unstained showing that staining in (A) was specifically due to Dnmt1o. (C) Transmitted light image of oocyte shown in (B). White scale bars represent 10  $\mu$ m.

meiosis the level of Dnmt1s in spermatocytes is high but a reduction in the level of the Dnmt1 enzyme has been observed in pachytene stage spermatocytes (Jue *et al.* 1995). This is due to the expression of an alternatively spliced version, *Dnmt1p*, which does not appear to be translated. Although Dnmt1s within the sperm is normally found in both the nucleus and the cytoplasm, it is concentrated at nuclear foci during some stages of meiosis and it may be that this correlates with the laying down of paternal imprints (Jue *et al.* 1995). Dnmt3l is expressed in the murine testes from E12.5 in non-dividing prospermatogonia with peak expression seen at the time of birth, after which there is a dramatic postnatal reduction in expression level (Bourc'his & Bestor 2004, La Salle *et al.* 2004). Dnmt3a expression in the testis is raised before birth and during early postnatal life, in contrast to the level of Dnmt3b expression which is lower during embryonic life and rises postnatally (La Salle *et al.* 2004). Mice lacking *Dnmt3l* have smaller testes, and by adulthood there are virtually no spermatozoa present, resulting in sterile animals (Hata *et al.* 2002). *Dnmt3l* is required if normal meiosis and silencing of retrotransposons is to occur (Bourc'his & Bestor 2004). The loss of *Dnmt3a* results in a similar although less extreme phenotype than that seen in the *Dnmt3l* KO mouse (Hata *et al.* 2002). More recently, the male *Dnmt3a* conditional KO was created, with no germ cell Dnmt3a expression but with somatic cell levels maintained (Kaneda *et al.* 2004). Spermatogenesis is severely impaired in these mice so that by 11 weeks of age there are no spermatozoa in the testis, demonstrating a vital role for *Dnmt3a* in this process. Offspring from these conditional KO males have errors in the methylation of some paternally imprinted genes.

### Fertilisation and early embryo development

As shown in Fig. 1, the fertilised embryo contains methylated DNA, some of which will be located in imprinted genes (both maternal and paternal) while the majority of

the DNA methylation will be positioned on non-imprinted sequences (again of both maternal and paternal origin). Early on in embryo development, the embryo loses its methylation at the non-imprinted DNA sequences; it now appears that this DNA methylation is lost in a parent-of-origin specific order, at least in some species. Imprinted genes are resistant to these early demethylation processes. The embryo's germ cells will lose methylation of the imprinted genes during gonadal development, while somatic cells maintain these methylation patterns throughout embryonic development and, in the main, throughout the life of the newly formed organism (although imprinted patterns are lost or altered in some tissues, such as the liver; McLaren & Montgomery 1999).

In some species (such as the mouse), the paternal genome is actively demethylated immediately after fertilisation (Oswald *et al.* 2000). The occurrence and degree of this demethylation appears to be species specific, and its regulation is currently unknown. Cross-fertilisation using gametes from several species of animal has shown that, although there are sperm characteristics which affect the degree of demethylation, the main factor determining whether the paternal genome becomes demethylated is oocyte specific (Beaujean *et al.* 2004). In the search for the factor responsible for this post-fertilisation active demethylation, MBD2 was proposed as a candidate after an *in vitro* study by Bhattacharya *et al.* (1999). However, a subsequent study utilising *MBD2*<sup>-/-</sup> oocytes has found that the rapid demethylation of the paternal genome still occurs in its absence (although this result does not eliminate the possibility of redundancy; Santos *et al.* 2002). The maternal genome undergoes passive demethylation which is slower to occur and is linked to the replication of DNA in the absence of any maintenance methylase activity. Although the general trend after fertilisation is for non-imprinted sequences to undergo demethylation, there does appear to be some specific incidences of *de novo* methylation such as the DMRs of the *Dnmt1o* gene in the one-cell to blastocyst stage embryo (Ko *et al.* 2005).



*Dnmt1o* remains localised to the cytoplasm of the embryonic cells at all pre-implantation stages with the exception of the eight-cell embryo. During this stage, the protein has been shown to translocate to the nuclei, where it is thought to play a role in maintaining the methylation level of imprinted genes. *Dnmt3a* is expressed by pre-implantation embryos and there is no evidence of the protein being excluded from the nucleus at any developmental stage (Ko *et al.* 2005). Although *Dnmt3b* is not transcribed in the pre-implantation embryo the protein is present at all stages from the one-cell to the blastocyst stage; while mainly localised to the cytoplasm, it is not fully excluded from the nucleus (Ko *et al.* 2005).

Until recently, embryos which consisted of two maternal or two paternal genomes were unable to develop to term. Work examining the competence of parthenogenetic embryos found they were able to develop at best until E9.5. By creating an embryo with one set of chromosomes from a fully grown and the other from a non-growing oocyte, Kono *et al.* (1996) showed that embryo development could be extended to E13.5. It was thought that this increase in the length of time the embryo survived was due to the ability of the non-growing oocyte chromosomes (with, therefore, no female imprints yet laid down) to partially compensate for the lack of a paternally imprinted set of chromosomes. Parthenotes which died at E9.5 and E13.5 had incorrect biallelic expression of the normally monoallelic *H19* gene. The most recent work by Kono *et al.* (2004) has resulted in the birth and survival of a viable parthenote. This was achieved using non-growing oocytes from a transgenic mouse with a 13 kb deletion of *H19*. The mouse had *H19* expression consistent with that normally seen from the paternal genome. As *H19* in turn influences the expression of the imprinted *Igf2* gene, the embryo had monoallelic expression of both *H19* and *Igf2* (from the wild-type fully grown oocytes only), as would be the case in normal embryos. The fact that normal embryo development is possible after the female genome is altered to more closely resemble the genomic imprinting pattern of the male genome demonstrates the importance of this mechanism in controlling development, reinforcing the idea that the presence of mammalian genomic imprinting might act as a barrier to parthenogenesis (see above).

### Errors in genomic imprinting

With genomic imprinting being a basic mechanism clearly vital for many aspects of development, there are, not surprisingly, many instances of developmental defects due to imprinting errors whether occurring naturally or during human intervention (Table 1).

### Assisted reproductive techniques

In recent years, there has been increasing concern that children conceived with the aid of ARTs could have an increased occurrence of disorders linked to imprinting

problems. At the turn of the century, two studies (Cox *et al.* 2002, Orstavik *et al.* 2003) reported the occurrence of three children conceived using intra-cytoplasmic sperm injection (ICSI) with Angelman syndrome (AS), a neurological disorder characterised by developmental delay and seizures, suggesting that the risk of AS may be increased by the use of ICSI. The fear of such a link was then increased with three studies that examined patients with Beckwith–Wiedemann syndrome (BWS) to see if a higher than expected proportion of these cases came from ART babies; all studies found a disproportionate number of such cases (DeBaun *et al.* 2003, Gicquel *et al.* 2003, Maher *et al.* 2003). BWS is characterised by both pre- and postnatal overgrowth and defects of the abdominal wall. Children who had been conceived using ART and suffered from BWS had the methylation status of their *H19* and *LIT1* genes established, with only one of the identified children demonstrating normal methylation patterns on both these genes. Data suggest that ART results in a three- to sixfold increase in the incidence of the normally rare BWS, although some of the studies may in fact be underestimating the true risk (DeBaun *et al.* 2003).

The cause of the link between ARTs and imprinting disorders is currently unknown. It could be due to some aspect of the ARTs involved. There is a wide range of different ARTs which are now routinely used within clinics. Techniques might expose one or both of the germ cells to an altered hormonal regime *in vivo*, a period of time in culture or mechanical manipulation. Any such alterations to the normal environment of the oocyte or sperm could result in changes to some aspect of their imprinting mechanisms. Alternatively, it could be due to some error within the germ cells used, bearing in mind that couples seeking to use ARTs have reduced natural fertility.

The potential problems do not end with the germ cells; the pre-implantation embryo is also often exposed to a period of culture which could again alter the epigenetic reprogramming known to occur at these early stages. One such example is abnormal biallelic *H19* expression of mouse embryos cultured in Whitten's media (Doherty *et al.* 2000). It is not just in humans and mice that potential problems with imprinting have been seen. In large domestic mammals such as sheep and cattle, large offspring syndrome (LOS) was identified when embryos had been exposed to some time in culture (Young *et al.* 1998). Further investigations into LOS in sheep have identified changes in the expression level of the imprinted gene, *IGF2R*, due to epigenetic changes (Young *et al.* 2001). Similar overgrowth problems seen in mice and humans are often caused by errors in several imprinted genes including *Igf2* and *H19* (Eggenschwiler *et al.* 1997), suggesting that other genes responsible for fetal growth and development could be involved in LOS.

If sperm used for *in vitro* fertilisation have lowered global methylation levels there is no alteration in either fertilisation rate or in early embryo quality; however, there is a reduction in pregnancy rate, demonstrating the importance

**Table 1** Diseases and syndromes which result from problems to the imprinting mechanisms or from errors in the imprinting of genes.

Disorder	Affected genes	Phenotype	Art link?
Angelman syndrome	Chromosome 15 – maternal copy, loss of <i>SNRPN</i> imprinting	Mental retardation, ataxic gait, seizures, sociable disposition	Yes
Autism	Unknown X-linked gene (not always connected to imprinting)	Impaired language development, problems with social and motor skills	
Beckwith–Wiedemann syndrome	11p15 region – altered expression of <i>IGF2</i> , <i>H19</i> and <i>LIT1</i>	Undescended testes, large newborn, seizures, abdominal wall defects	Yes
Cancer	Variable, e.g. <i>IGF2</i> in lung cancer (not always connected to imprinting)	Tumours	
ICF (immunodeficiency, centromeric region instability and facial anomalies syndrome)	<i>DNMT3B</i>	Immune problems, facial anomalies, growth retardation	
Paraganglioma	Paternal mutations <i>SDHA</i> ( <i>PGL1</i> ) and <i>PGL2</i>	Glomus tumours of the parasympathetic ganglia mainly in the head and neck region, tend to be slow growing and benign	
Prader–Willi Syndrome	Chromosome 15 – paternal copy	Undescended tests, mental retardation, short stature, obesity small hands and feet	
Pre-eclampsia	Not yet defined	Serious complication of pregnancy	
Pseudohypoparathyroidism type IA (Albright hereditary osteodystrophy)	Imprinted <i>GNAS</i> cluster	Parathyroid hormone resistance, short stature, round face and short hand bones	
Pseudohypoparathyroidism type IB	Imprinted <i>GNAS</i> cluster	Parathyroid hormone resistance localized to renal system, causing hypocalcaemia and hyperphosphataemia	
Rett syndrome	MeCP2	Childhood neurodevelopmental disorder mainly affecting females. Loss of motor function and mental retardation	
Silver–Russell syndrome	Cases which are imprinting related – chromosome 7	Short stature, excessive sweating, triangular face, inward curving 5th fingers and coloured spots on the skin	
Transient neonatal diabetes	An imprinted gene at 6q24. Candidates are <i>ZAC</i> & <i>HYMAI</i>	Growth retardation and diabetes which develops during the first 6 months of life but corrected by 18 months	
Turner syndrome	Complete or partial loss of second X chromosome	Affects females – short stature, social problems and ovarian failure	
Wilms' tumour	<i>IGF2</i> loses imprinting	Childhood kidney tumour	

of normal gamete DNA methylation on embryo development and ultimately ART outcome (Benchaib *et al.* 2005). There is recent evidence that sperm obtained from males with low sperm counts due to abnormal spermatogenesis have incorrect genomic imprinting (Marques *et al.* 2004), although such sperm can then be used, for example, in ICSI. Marques *et al.* (2004) found that, although the maternal imprints had been erased from all sperm, the paternally methylated *H19* gene was under-methylated in some sperm from the oligozoospermia donors. Any embryo derived from one of these hypomethylated sperm could have inappropriate expression of the imprinted *H19* and *IGF2* genes, the effect of which is not known.

### Cloning

Studies investigating the failure of cloned animals have also turned their attention to the role of genomic imprinting. The fact that many of the errors seen in cloned animals have epigenetic causes has been demonstrated by examining the offspring of cloned mice. These cloned mice were obese but this trait was not passed onto the offspring, demonstrating that this was not a genetic error but due to epigenetics. This finding is important as it suggests

that, despite any problem in the cloned animals, it is possible that their germ cells are able to correctly undergo genomic imprint reprogramming (Tamashiro *et al.* 2002). In bovine cloned embryos, it has been found that the levels of methylation in the cells of the embryo are higher than normal at the four-cell and eight-cell stages. Although there is initial demethylation of the donor genome, passive demethylation does not occur to the level seen in normal embryos. In addition to a reduction in the amount of demethylation, there also appears to be inappropriate *de novo* methylation occurring at early stages of embryo development (Dean *et al.* 2001). It is also possible that errors in the Dnmt enzymes normally present in the early embryo could account for alterations in methylation seen in these embryos. Analysis of cloned mouse embryos shows inappropriate presence of Dnmt1s within the pre-implantation embryo; this transcript of Dnmt1 is never present in normal embryos. It was also observed that at the eight-cell stage, when Dnmt1o would normally translocate into the nuclei of embryonic cells, some nuclei within each embryo were devoid of any Dnmt1 transcript, suggesting that these cells are unable to maintain normal methylation patterns (Chung *et al.* 2003).



## Disease

In some cases, imprinting errors can occur which, although not embryo lethal, cause abnormal physiological processes and lead to disease. Such diseases can arise when any imprinted gene becomes hypermethylated or hypomethylated. Effects are not always limited to the loss of function of a single gene, as some imprinted genes affect the expression of other genes, such as *H19* and *IGF2*. The linked Prader–Willi syndrome (PWS) and AS are examples of disorders that can occur when correct imprinting is lost. A loss of a currently unidentified imprinted gene results in PWS when the deletion is paternally inherited, whereas the same errors cause AS to develop when maternally transmitted (Moncla *et al.* 1999). Other examples of diseases which result after incorrect imprinting include BWS, Silver–Russell syndrome and transient neonatal diabetes.

Disease can also result from defects in mechanisms regulating imprints. One of the key groups of enzymes with a role in genomic imprinting are the Dnmts which are responsible for the addition of methyl groups to the DNA. When problems arise within this aspect of the imprinting mechanism it can lead to disease in the individual. One such example is immunodeficiency, centromeric region instability and facial anomalies syndrome which is a result of a mutation in *DNMT3B* (for review see Ehrlich 2003).

Another major component of the imprinting mechanism is the family of methyl-binding domain proteins. MeCP2 is a protein which contains a methyl-binding domain. It has a role in controlling the transcription of imprinted genes through its ability to bind to methylated DNA. The importance of this protein for normal development and physiological function is demonstrated by Rett syndrome which occurs when *MECP2* is mutated (Amir *et al.* 1999).

There are some diseases with multiple causes which only in some cases involve errors to the imprinting mechanism or alterations to imprinted genes. Cancer is one such disease, with some cases of cancer being identified as having a cause linked to genomic imprinting while many other incidences of the disease occur because of unrelated problems. In some instances, human tumour cells have been found to overexpress one or more of Dnmt1s, 3a and 3b, with the largest upregulation occurring to Dnmt3b (Robertson *et al.* 1999). These results support the previous observations of abnormal methylation levels seen in tumour cells. One cancer which demonstrates such raised Dnmt levels is acute myelogenous leukaemia; it may be that this overexpression of the Dnmt enzymes accounts for the hypermethylation and silencing of an important tumour suppressor gene (Mizuno *et al.* 2001).

## Conclusion

Genomic imprinting is a gene transcription control mechanism which is vital for normal healthy offspring. Although in recent years there has been a huge volume of

work undertaken to elucidate the mechanisms behind genomic imprinting there are still many unanswered questions. Recent data have demonstrated that there are species differences in the imprinting mechanism which still need to be fully explored but could have implications for the success of cloning attempts. Additionally, knowledge of genomic imprinting may aid the understanding of some human diseases and offer potential therapies. The field of ART will also benefit from a greater understanding of genomic imprinting, resulting in improved techniques with an increased success rate and, most importantly, a safer outcome.

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