Epigenetics and the germline

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Abstract

Epigenetic processes affect three stages of germline development, namely (1) specification and formation of primordial germ cells and their germline derivatives through lineage-specific epigenetic modifications, in the same manner as other embryonic lineages are formed, (2) a largely genome-wide erasure and re-establishment of germline-specific epigenetic modifications that only occurs in the embryonic primordial germ cell lineage, followed by re-establishment of sex-specific patterns during gametogenesis, and (3) differential epigenetic modifications to the mature male and female gamete genomes shortly after fertilisation. This review will detail current knowledge of these three processes both at the genome-wide level and at specific imprinted loci. The consequences of epigenetic perturbation are discussed and new in vitro models which may allow further understanding of a difficult developmental period to study, especially in the human, are highlighted.

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Epigenesis

In terms of regulation of gene expression in the germline, epigenesis can be defined as ‘alterations in DNA function without alterations in DNA sequence’ (Jones & Takai 2001). In somatic cells, DNA exists in a nucleosomal form, with chromatin formed in conjunction with core and linker histones (Li 2002). The complex pattern of silenced/expressed gene regions that exists within the genome of a single cell type is tightly regulated by epigenetic modifications, either to the histones or to the DNA itself (Felsenfeld & Groudine 2003, Jaenisch & Bird 2003). Although the exact ontogeny of events remains controversial (Li 2002), it is likely that when regions of active chromatin are silenced, histone deacetylases remove acetylation from the histones. If the genome is to be permanently silenced in this region (rather than just transiently, e.g. during the cell cycle), then methyl groups are transferred to cytosine residues present in a CpG base pair configuration through the action of the de novo methyltransferases, DNMT3a and 3b. During subsequent cellular generations, this methylation is faithfully recapitulated by the action of the maintenance methyltransferase, DNMT1 (Li 2002). The linking of DNA methylation and histone deacetylation in specific genomic regions appears to confer a powerful mechanism for long-term gene silencing (Meehan et al. 2001, Bird 2002, Sutherland & Costa 2003). It is emerging that the trigger for de novo methylation of DNA involves a series of histone modifications other than deacetylation, with methylation of histone H3 at the lysine 9 position (H3-K9) strongly correlating with DNA methylation (Felsenfeld & Groudine 2003, Jaenisch & Bird 2003).

There are several consequences of the epigenetic methylation of DNA. One of the major functions of DNA methylation appears to be to permanently silence the large proportion of ‘junk’ DNA, repetitive sequences that have entered our genomes throughout the course of evolution, mainly by viral transfection (Jaenisch 1997, Yoder et al. 1997). Forty-five percent of the human genome consists of viral retrotransposons and endogenous retroviruses (Lander et al. 2001), repeat sequences that are capable of moving around the genome and/or causing instability and inappropriate expression of local genes if not kept in check by strong silencing mechanisms. The highly abundant Satellite repeat sequences are permanently located in the hypermethylated centromeric heterochromatin regions, while there are many dispersed repeats that exist within the more active euchromatic regions. The complex epigenetic regulation of gene expression is also thought to have been an evolutionary prerequisite to multicellularity (Jablonka & Lamb 1998), allowing development of lineage-specific gene expression patterns within organisms whose cells all contain the same genetic code. Thus, as each new lineage forms within the developing embryo, a unique pattern of silenced/expressed regions is set up (Bird 2002, Shiota et al. 2002).
**Formation of the germline**

**Mouse**

Until recently, it was considered that the germline of mammals was not a consequence of this epigenetic developmental process but that, like several lower organisms, a subset of the oocyte cytoplasm known as the germplasm was already set-aside to form the germ cells prior to fertilisation (reviewed by Johnson et al. 2003a,b). In fact, cell fate mapping revealed the origin of primordial germ cells (PGCs) in mouse to be in the proximal epiblast of the embryo (Lawson & Hage 1994), with germ cell competence induced by bone morphogenetic protein (BMP)4 from the extraembryonic endoderm (Saitou et al. 2002). At 6.25 days post-coitum (dpc), this BMP4 induction primes the proximal epiblast to become interferon responsive (Lange et al. 2003) and interferon induces *fragilis* gene expression, defining the portion of embryonic mesoderm with germ cell competence (Saitou et al. 2002). At 7.0 dpc, PGCs arise from a population of around 100 cells (Ginsburg et al. 1990) and a subset of high-*fragilis* expressing cells then induce *stella*, the product of which represses the homeobox gene expression pathway that would induce somatic cells such as those of the haemopoietic system and instead restricts a germ cell fate (Saitou et al. 2002). By 8.0 dpc the PGCs are distinct at the base of the allantois (Nagy et al. 2003) and then migrate to the embryonic hindgut (Tam & Snow 1981). By 8.5 dpc, they further migrate through the embryonic mesentery, linking through E-cadherin- (Bendel-Stenzel et al. 2000) and β1 integrin (Anderson et al. 1999)-mediated cellular processes before arriving at the gonadal ridge at 11.5 dpc. During the migratory period the PGCs begin to proliferate, completing formation of around 50000 cells by two days after entry into the gonadal ridge (Donovan et al. 1986, Nagy et al. 2003). PGC proliferation during migration and before reaching the gonadal ridge is controlled by stem cell factor (SCF), c-Kit receptor tyrosine kinase and c-Kit ligand (Readhead & Muller-Tidow 2002). PGCs in the gonadal ridge express *vasa* and *dazl* (Noce et al. 2001, Saunders et al. 2003), they become non-motile and then enter first meiotic prophase (female gonocytes) or mitotic arrest (male gonocytes). The default pathway in mammalian gonadal ridges is to form an ovary; only when expression of the *sry* gene is initiated from a Y chromosome will the testis-specific pattern of gene expression be initiated (Knowler et al. 2003). A range of factors, including Gas-6, Gata-4, Zfx (females), Zfy (males), SF-1 and Wt-1 are involved in the differentiation of PGCs into male or female gonocytes depending on the XX or XY composition of the embryo and male-specific expression of *sry* (Knowler et al. 2003). It is not until the onset of puberty that meiosis I-arrested female gonocytes and mitosis-arrested male gonocytes continue division and become haploid (Picton et al. 1998, Donovan & de Miguel 2003).

**Human**

Due to obvious difficulties and ethical restrictions in obtaining experimental material, much less is known regarding the gene expression patterns regulating germ cell fate in humans. Sadler (2004) recently reported that human PGCs are formed in the epiblast during week 2 of development. At week 4, the cells begin to migrate from the yolk sac towards the presumptive gonads (Goto et al. 1999, Larsen 2001, Sadler 2004). By the end of week 5, the PGCs arrive in the gonadal ridge (Sadler 2004) and by 10 weeks, female germ cells have entered meiosis while male germ cells continue mitosis until 16–18 weeks of gestation (Goto et al. 1999). In both sexes, mature germ cells are not formed until puberty, as in the mouse.

**Epigenetic changes associated with germline specification**

After fertilisation, the genomes inherited from both sperm and oocytes undergo a cycle of erasure of most of their associated DNA methylation, with lineage-specific patterns of *de novo* methylation occurring during or after gastrulation. Like other newly-formed lineages, primordial germ cells also undergo this phase of *de novo* methylation (Hajkova et al. 2002).

While the epigenetic regulation of this germline specification programme in specific genes has not yet been examined, evidence for specific epigenetic modifications of germline genes has surprisingly come from the study of a variety of adult tumours. Loriot et al. (2003) identified a number of germline genes that were aberrantly reactivated in tumours and demonstrated that reactivation was associated with demethylation of the promoter regions. Interestingly, many of these genes were specifically expressed in spermatogonia, the testis stem cells, rather than any later gamete stages. When lymphoid cell lines were treated *in vitro* with the DNA methylation inhibitor 5-azadeoxycytidine, these spermatogonial genes could also be reactivated *in vitro*.

More recently, both the *oct4* (Gidekel & Bergman 2002, Hattori et al. 2004) and *sry* genes (Nishino et al. 2004) involved in germ cell pluripotency and gonadal sex determination respectively, have been shown to have their temporal and tissue-specific patterns of gene expression regulated by DNA methylation, with regulatory regions of these genes undergoing a brief period of demethylation concurrent with gene expression. The methylation status of PGC specification genes such as *fragilis* and *stella* is not known; however their temporally restricted expression might predict regulation by DNA methylation/demethylation as in *sry* and *oct4*. A homologue of *stella* has recently been described in the human genome (Payer et al. 2003) that is also expressed in human oocytes (Goto et al. 2002), suggesting conservation of function.

The epigenetic regulation of spermatogenesis at least is beginning to be investigated, with mice null for *Dnmt3a,*
Dnmt3l and histone methyltransferases all disrupting various phases of meiosis (Li 2002). Between the preleptotene and pachytene stages of spermatogenesis, there appears to be a coupling of histone H3-K9 methylation and histone deacetylation which has been suggested to suppress global gene expression when spermatocytes are undergoing meiosis (Li 2002).

**Germline epigenetic reprogramming**

In addition to requiring epigenetic modifications to form the germline, the PGCs uniquely undergo an extra and very dramatic wave of epigenetic reprogramming that most other lineages do not undergo. This process is thought to be essential for the generation of new cells that will allow totipotency in the preimplantation embryo, for ensuring that both sexes acquire an equivalent epigenetic state prior to the differentiation of sex-specific gametes and imprints (see below) and to allow erasure of epimutations that adult cells (presumably including gametes) can inherit during their lifetime. It is of obvious evolutionary advantage to remove such errors in the germline prior to forming a new generation. Although aberrant inheritance of epimutations is known to occur in plants, recent studies in mice suggest that this phenomenon may also occur in mammals and may underlie the inheritance of some disease phenotypes or susceptibility (Rakyan et al. 2001, 2003).

**Reprogramming of non-imprinted genes**

Within one day of entering the genital ridge, both male and female mouse PGCs actively erase their methylation component in a number of sequences (Hajkova et al. 2002). By 12.5 dpc, methylation of the single copy genes *alpha actin* and *mylce* are erased and it is assumed that this is representative of other single copy sequences in the genome. Of interest, however, is that some repetitive elements do not entirely erase their methylation prior to mitotic/meiotic arrest (Walsh et al. 1998, Hajkova et al. 2002), while others undergo substantial demethylation (Lane et al. 2003).

Within the gonad, sex-specific epigenetic patterns are then re-established at different developmental stages (Hajkova et al. 2002). In the male mouse, remethylation begins before birth (15.5–18.5 dpc) in prospermatogonia and is completed prior to the end of meiotic pachytene after birth. Using an antibody raised against 5-methylcytosine, the 15.5 dpc testis showed no genome-wide labelling of methylated DNA in the gonocytes, only in the somatic Sertoli cells, whereas by 18 dpc spermatogonia were also labelled (Coffigny et al. 1999). This study observed that male germ cells always possess hypomethylated heterochromatin relative to somatic cells, whereas their euchromatin passes from a demethylated to a strongly methylated status between days 16 and 17 postcoitum. This is consistent with the relative undermethylation of centromeric Satellite DNA in germ cells (Ponzetto-Zimmermann & Wolgemuth 1984, Sanford et al. 1984), although the significance of this is not yet understood. In the mouse, even in mature gametes, the pattern of methylation is germline specific so that sperm DNA is globally hypermethylated compared with oocyte DNA, but both gametes are hypomethylated relative to somatic tissues (reviewed by Constancia et al. 1998). Recently, a study of 1500 CpG island regions, associated with gene promoters, found that 30 methylated loci detected in mature spermatozoa were not detected in embryonic germ (EG) cells, indicating widespread epigenetic change associated with germline differentiation (Shiota et al. 2002). Sixty-six percent of the gene-associated regions analysed were unmethylated, suggesting that the global hypermethylation previously reported in sperm may be due to abundant repeat sequences and do not reflect the majority of PGC genes. However, artefactual results arising from derivation or culture of EG cells compared with PGCs cannot be ruled out.

Remethylation of the female germline has not been examined at the global level and so information on the timing of this process is largely only available for imprinted genes as discussed below. However, it is known that both growing and mature mouse oocytes have lower Satellite and dispersed repeat methylation than sperm or somatic cells (Monk et al. 1987, Sanford et al. 1987, Howlett & Reik 1991).

**Reprogramming of genomic imprints**

Imprinted genes comprise a small subset of the genome (perhaps 100 out of the total 30 000 genes (Miozzo & Simoni 2002) whose epigenetic reprogramming in the germline is imperative for subsequent normal development of the embryo. Genomic imprinting is the phenomenon that gives rise to differential expression of paternally and maternally inherited alleles of certain genes due to sex-specific epigenetic differences inherited from the germline. Thus, unlike most genes in our genome, which are either expressed or silenced from both parentally inherited alleles (biallelic expression), monoallelic expression of imprinted genes occurs in a tissue- and developmental stage-specific manner during development (Lyle 1997, Miozzo & Simoni 2002). Genomic imprinting has been reported in several mammalian species – humans, mouse, rat, sheep, marsupials etc (Miozzo & Simoni 2002 and see http://cancer.otago.ac.nz:8000/table.html) with monoallelic expression appearing restricted to the embryonic and fetal periods. Those imprinted gene loci that express in post-natal tissues invariably show biallelic expression (Moore 2001, Reik & Dean 2001). Since monoallelic expression affects the dosage of the mRNA and resulting protein, the effect of the imprinting process is to limit the effect of the gene product.

The monoallelic expression of imprinted genes involves significant differences in allelic chromatin configuration due to differential DNA methylation of cytosine residues, phosphorylation, histone acetylation and methylation of
histone proteins as well as the modification and assembly of regulatory protein complexes on DNA (Meehan 2003). These imprinted differences between the egg and sperm are then transmitted to the somatic cell lineages that form after fertilisation.

In the germline, DNA methylation is so far the most widely studied epigenetic process associated with genomim imprinting, although whether it represents the primary ‘imprint’ distinguishing the parental alleles is currently under debate. Differential methylation arises in specific gene regions known as differentially methylated regions or DMRs. Two types of DMRs are known: primary DMRs comprising DNA sequences methylated differentially in oocytes, and sperm and secondary DMRs that form after fertilisation. Allele-specific methylation patterns are considered to be established and maintained due to the presence of repetitive sequence regions near DMRs (Constancia et al. 1998, Lucifero et al. 2004). The restriction of de novo methylation to specific imprinted genes in the developing germline is thought to involve the CCCTC-binding factor (CTCF) boundary element (Fedoriw et al. 2004). When the zinc finger CTCF protein binds to its target binding site in imprinted genes (if the site is unmethylated), the complex acts to partition the genome into active or inactive chromatin domains (Burgess-Beusse et al. 2002) and to prevent further recruitment of DNA methylation and aberrant silencing of neighbouring regions. RNA interference-induced deficiency of CTCF resulted in an increase in methylation at the H19 DMR region and a decrease in development competence of the oocytes. Thus CTCF appears to protect the maternal DMR (at least for H19) from de novo methylation during oocyte growth (Fedoriw et al. 2004). A male germline-specific homologue of CTCF, BORIS, is discussed below.

The allelic methylation of imprinted genes appears to occur through a unique combination of methyltransferase enzymes, and methylation coincides with the presence of high levels of DNA methyltransferases in the oocyte nucleus (Constancia et al. 1998, Lucifero et al. 2004). Dnmt3l is similar to the de novo methyltransferases, Dnmt3a and 3b, in many structural domains except that it lacks methylation catalytic activity. Dnmt3l interacts and colocalises with Dnmt3a to modulate de novo methylation of imprinted genes in the female gamete (Chedin et al. 2002, Hata et al. 2002). Male mice null for Dnmt3l produce spermatogonia unable to undergo differentiation and meiosis while female mutant mice were unable to methylate sequences that are normally methylated maternally (Bourc’his et al. 2001b). The effect of lack of Dnmt3l was specific to imprinted regions, with global genome methylation levels unaffected. The lack of maternal methylation imprints resulted in biallelic expression of genes that are normally only paternally expressed (Bourc’his et al. 2001b). Dnmt1o is the oocyte-specific form of Dnmt1, involved in methylation of maternal imprints in the 8-cell stage mouse embryo (Doherty et al. 2002).

It has also been suggested that different chromatin regulatory factors are present in the two forming germlines, that might promote different chromatin organisations which are either conducive to or inhibit DNA methylation (Feil & Kosola 1999, Lucifero et al. 2004). Paulsen and Ferguson-Smith (2001) report that DMRs do not necessarily retain a methylation pattern acquired during gametogenesis and Igf2 and H19 DMRs do not represent primary imprints (Olek & Walter 1997, Oswald et al. 2000). It appears that although methylation imprints are erased in PGCs, other epigenetic regulation factors may not be and it may be these factors that direct DNA methylation exclusively. While sex-specific forms of Dnmt1 have been described (Mertineit et al. 1998, Pradhan & Esteve 2003), other germline-specific epigenetic or chromatin remodelling factors have not been identified. However, clues to potential regulators may arise from the study of differential modifiers of the male and female pronuclei in the zygote, where sex-specific differences in binding of heterochromatin protein 1 (HP1) and histone H3-K9 methylation have been established (Arney et al. 2002, Cowell et al. 2002).

Support for the functional importance of methylation during oocyte growth comes from nuclear transplantation studies where parthenogenetic embryos containing one genome from neonate-derived non-growing oocytes (with no female imprints, thus mimicking a male genome) and the other from a fully grown oocyte (with fully developed female imprints) develop for a longer period than do normal parthenogenetic samples (Kono et al. 1996, Obata et al. 1998). Definitive proof that it is the lack of complete imprinting that is the cause of developmental failure in these embryos seemed to be provided recently from a study where deleting H19 in the non-growing oocytes (restoring normal expression of the imprinted fetal mitogen, Igf2) allowed full-term survival of a single non-growing/fully grown oocyte pronuclear hybrid (Kono et al. 2004). However, until this is repeated and demonstrated not to be just a chance event, the significance of the parthenogenetic birth is still unclear (Moore & Ball 2004).

**Imprinting inheritance**

Since epigenetic imprints are fully established in the embryo by the time of gastrulation (Reik et al. 2001), all subsequent cell lineages, including PGCs, will contain imprinted loci that are differentially marked on both alleles. Thus, when germ cells undergo meiosis and become haploid, this ‘imprint’ must be able to be erased and reversed so it can be transmitted to the next generation in a sex-specific manner (Fig. 1). If this process did not occur, half of all sperm (or oocytes) ultimately formed within an individual could potentially have the wrong parental imprint, risking the production of all female (parthenogenetic) or all male (androgenetic) embryos at fertilisation (Fig. 2). Studies on parthenogenetic mice (Surani et al. 1984, Kono et al. 1996) and sheep (Feil et al. 1998, Hagemann et al. 1998),
both of which die in early/mid gestation, have shown that correctly established imprints from both sexes are required for normal fetal development.

**Erasure of imprints**

Several studies have now demonstrated that the erasure of at least methylation imprints occurs in the germline shortly after the PGCs enter the gonadal ridge. PGCs migrating to the genital ridge around 9.5–10.5 dpc are reported to have the allele-specific imprints characteristic of other somatic lineages at this time (Ueda et al. 2000, Surani 2001), although at least some erasure of imprints may already be initiated by 10.5 dpc (Yamazaki et al. 2003). In support of this, Igf2r transgenic mice expressing green fluorescent protein specifically in the germline begin to demethylate some CpG sites within DMR2 at 9.5 dpc in some migrating PGCs before the cells colonize genital ridges (Sato et al. 2003). However, progression of Igf2r demethylation is more rapid after colonization of the genital ridges. Within a day of entering the genital ridge (10.5–11.5 dpc), analysis of a range of imprinted genes has revealed that erasure is completed and is maintained until at least 13.5 dpc (Kafri et al. 1992, Hajkova et al. 2002, Yamazaki et al. 2003).

**Re-establishing imprints in the female**

After erasure of germline methylation imprints, differentiating germ cell genomes must become maternalised or paternalised depending on germ cell sex, and this must occur before the onset of meiosis. In the female germline, imprints are re-established in growing oocytes. Various imprinted genes receive an ‘imprinting mark’ asynchronously at particular stages during oocyte meiotic prophase I, during the primordial to antral follicle transition (Hajkova et al. 2002, Obata & Kono 2002). Disruption of this primary imprinting process can lead to altered expression patterns of imprinted genes during embryogenesis.

**Re-establishing imprints in the male**

The timing of methylation re-establishment in imprinted genes is less clear for the male germline. However, it appears that paternal imprints are established early in diploid gonocytes (reviewed by Lucifero et al. 2002). Murine H19 methylation, for example, appears to be initiated in prenatal prospermatogonia and is completed postnatally by the pachytene stage of meiosis (Ueda et al. 2000). Manning et al. (2001) examined the 15q11–13 imprinted
chromosome region associated with Prader-Willi/Angelmann syndromes and revealed correct paternally established imprints in ejaculated spermatozoa, elongated spermatids and amplified round spermatids, indicating completion of imprinting by the time of haploidisation. Examination of embryos produced by intracytoplasmic sperm injection (ICSI) of round spermatids also revealed normal imprinting of Mash2, Igf2r and mostly of H19, also confirming completion of paternal imprinting prior to meiosis (Shamanski et al. 1999). Zalduendo et al. (2001) examined methylation of the maternally imprinted gene, U2af1-rs1, in three stages of male germ cell line development. Methylation increased as developmental time increased from unmethylated PGCs (or at least in the EG1 cell line derived from 8.5 dpc embryos), through spermatogonia and spermatocytes, Sertoli cells and finally mature sperm cells (12- to 16-week-old mice). DNAase 1 sensitivity was also higher in both the PGC-derived EG line and in spermatogonia than in somatic Sertoli cells, suggesting a more open chromatin structure in this imprinted region in the germline.

Another intriguing finding in the re-establishment of imprints has recently been uncovered. It appears that for both Snrpn and H19, acquisition of the paternal imprint occurs at different times during spermatogenesis, depending on whether the spermatogonia originated from a male- or female-derived allele in the diploid PGCs (Davis et al. 2000, Lucifero et al. 2004). Thus, for at least a small subset of genes, these observations challenge the concept of epigenetic reprogramming in the germline resulting in complete equivalence of the germlines prior to meiosis.

The male germline has recently been shown uniquely to express a parologue of CTCF, known as Brother of the Regulator of Imprinted Sites (BORIS; Loukinov et al. 2002). BORIS is expressed in a mutually exclusive manner with CTCF during male germ cell development, and the erasure of methylation marks during male germine development is associated with dynamic up-regulation of BORIS, as well as down-regulation of CTCF. Remethylation of DNA in round spermatids is associated with the subsequent silencing of BORIS and reactivation of CTCF. Thus BORIS could be associated with demethylases that participate in the erasure of methylation marks, and BORIS–CTCF switching may be intimately linked with the co-expressed testis-specific histone H3 lysine methylase, Suv39h1, and propose that histone methylation may mark the region-specific DNA for de novo methylation which is then mediated by BORIS or CTCF.

**Reprogramming of imprints in the human germline**

No studies are yet available on the full ontogeny of germine epigenetic reprogramming in the human; however there is some information available for late-stage gametes and for preimplantation embryos. SNRPN is methylated in human late-stage germinal vesicle, metaphase I and metaphase II oocytes, and in 4-cell embryos (Geuns et al. 2003). H19 is unmethylated in fetal gonocytes, and methylated in spermatogonia, spermatozoa and preimplantation embryos. IGF2 shows monoallelic expression in the human blastocyst, indicating that differential methylation is at least complete by this stage (Lighten et al. 1997).

Kerjean et al. (2000) also showed that some paternal imprints are established during human spermatogenesis. H19 and PEG1 are unmethylated in fetal spermatogonia, therefore suggesting that all pre-existing methylation imprints are already erased by this stage. PEG1 remains unmethylated at all subsequent post-pubertal stages of spermatogenesis including mature spermatooza. H19 methylation, typically seen on the paternal allele, first appears in a subset of adult spermatogonia and is then maintained in spermatocytes, spermatids and mature spermatooza. Information is beginning to emerge on the DNA methyltransferases that may be associated with genomic imprinting in the human germline. A human homologue of the Dnmt10 gene has been identified in immature oocytes and early preimplantation embryos (Hayward et al. 2003), although the protein has never been examined. However, while Dnmt3l is expressed in mouse oocytes throughout oogenesis, in zygotes and in blastocysts, in humans, Dnmt3L appears only to be expressed after fertilisation, and therefore it has been proposed that either DNMT3L may not be involved in establishing imprinting in humans or that there are relative timing differences (Huntriss et al. 2004, Young & Beaujean 2004).

**Errors of epigenetic reprogramming**

Several experimental studies and naturally occurring disease or developmental defects are known to arise from epigenetic defects that may arise in the germline. These defects have been important in contributing to our understanding of the role of and the mechanisms underlying epigenetic phenomena. Here we will consider epigenetic defects that result from genome-wide errors as well as errors in specific loci.

Recently, a human recurrent hydatiform mole phenotype reminiscent of androgenetic extraembryonic tissue, has been described which results from an inherited failure to establish any imprints in the female germline (Judson et al. 2002). The effect of a lack of female imprints has also been demonstrated in nuclear transplantation experiments, using germ cells from various stages of development as diploid donor cells for nuclear transfer into enucleated oocytes (Yamazaki et al. 2003). Embryos thus ‘cloned’ from 10.5 dpc migrating male germ cells allowed fetal development to mid-gestation, while those from 11.5, 12.5, 13.5 and 15.5 dpc embryos or from gonocytes harvested from postnatal ovaries showed a relative developmental delay (Yamazaki et al. 2003).
Although no studies have examined disrupted imprinting in the early male germline, Marques et al. (2004) have very recently researched the idea that imprinting defects may be associated with disturbed spermatogenesis. MEST (PEG1) and H19 methylation were examined in sperm from normozoospermic and oligozoospermic patients and MEST maternal imprinting was correctly erased in all patients. However, methylation patterns for H19 were abnormal in 23 out of the 96 oligozoospermic patients tested. Most patients had both normally methylated and hypomethylated alleles in the same spermatozoan sample. Imprinted genes are very important in fetal, placental and behavioural development and monoallelic expression has mostly been identified in prenatal development (Reik et al. 2003). Their misregulation has been implicated in a variety of pathologies including sporadic, inherited and induced growth disorders (Falls et al. 1999, Miozzo & Simoni 2002, Walter & Paulsen 2003). Loss of imprinting (resulting in biallelic expression) at growth-related gene loci is well documented to produce growth defects in humans such as Beckwith-Wiedemann syndrome (BWS; Weksberg et al. 2003) or intra-uterine growth restriction (IUGR; Preece 2002), Large Offspring syndrome (LOS) in sheep and cattle (Young et al. 2001) and similar growth effects in genetically manipulated mice (Moore 2001). Other syndromes with more behavioural phenotypes associated with loss of imprinting include Prader-Willi and Angelman syndromes (Cassidy et al. 2000). In the case of mouse gene targeting experiments, it is clear that the effect arises from an embryonic defect, whilst in the case of LOS and the human syndromes, it is not clear whether germline or embryonic disruptions (or both) have occurred. Increased incidences of the imprinting defects, BWS and Angelman syndrome, have been reported in human-assisted reproduction technologies (ART) in comparison with normally conceived offspring (Gosden et al. 2003, Maher et al. 2003). This raises the possibility that ARTs such as in vitro maturation of oocytes, ovarian hyperstimulation, embryo culture and the use of sub-fertile sperm may introduce imprinting errors into conception and this now requires closer monitoring of infertility treatments (Young & Fairburn 2000, Young 2003).

A well-understood consequence of aberrant DNA methylation in somatic cells is the formation of tumours, where hypermethylation can silence tumour suppressor genes and hypomethylation can activate oncogenes (Szyf 2003). Teratocarcinomas are tumours containing tissues derived from all three of the germ layers - endoderm, mesoderm and ectoderm. These can arise due to uncontrolled PGC proliferation, perhaps due to ectopic expression of c-Kit or other genes involved in the growth of PGCs, and may be caused by PGCs which have gone astray during the migration from yolk sac to gonadal ridge (Donovan & de Miguel 2003). If epigenetic reprogramming is misregulated in PGCs, this may provide one possible mechanism for their transformation, although this has not yet been examined. The observation of ectopic PGCs when E-cadherin-mediated cell adhesion was blocked using antibodies (Bendel-Stenzel et al. 2000) reveals a mechanism which may allow stray cells to move to regions outside the gonad and potentially develop germ cell tumours, perhaps by being exposed to different epigenetic signals. Since many imprinted genes are known tumour suppressors or oncogenes involved in cell proliferation, they often show disrupted imprinting in a variety of adult tumours. Thus, it is of interest that Hernandez et al. (2003) observed opposite effects of the paternal and maternal genomes on proliferation, cell-cycle length, senescence, and tumour formation of androgenetic and parthenogenetic cells in culture.

Epigenetic alterations in DNA methylation in other genomic regions have also been reported, as a consequence of altered nutrition. A maternal hypermethylating diet during mouse pregnancy increased DNA methylation in repetitive sequences, promoting ectopic expression of the agouti gene (Waterland & Jirtle 2003). Since the hypermethylating diet in this study was applied during oogenesis, pregnancy and weaning, it is not possible yet to discern whether nutrients can specifically affect methylation of the germline, but the human health implications certainly make it a possibility that merits testing (Young 2001, Young et al. 2004).

Epigenetic reprogramming of gametes after fertilisation

The mouse genome undergoes two major phases of epigenetic reprogramming, once in the primordial germ cells and once in the preimplantation embryo. The mouse genome undergoes two major phases of epigenetic reprogramming once in the primordial germ cells and once in the preimplantation embryo (Fig. 3). Both involve genome-wide erasure of DNA methylation and then a period of locus-specific de novo methylation, but only in the PGCs are imprints erased and re-established (Santos & Dean 2004). After fertilisation in mice, during pre-implantation and early post-implantation stages, demethylation takes place; however, this does not affect the parental genomes to an equal extent, such that even in the embryo the two germlines exhibit differences in epigenetic reprogramming. Whilst the paternal pronuclear genome is rapidly demethylated in the newly fertilised egg, the maternal genome is gradually demethylated during the first cell cleavages (Mayer et al. 2000). Equivalent levels of hypomethylation of both parental genomes are reached by the 16-cell stage then the combined embryonic genomes are de novo methylated in the inner cell mass of the murine blastocyst (Santos et al. 2002).

Whilst the global demethylation of the paternal genome observed in the mouse embryo has also been reported in the rat, pig, cow (Dean et al. 2001) and human (Beaujean et al. 2004a) zygote, this does not occur in either the sheep or rabbit embryo (Beaujean et al. 2004a, Shi et al. 2004) and thus is not representative of all mammals.
Insights into these species differences have recently been provided by interspecies ICSI experiments, which have shown greater demethylating capacity of mouse ooplasm compared with that of the sheep, in addition to greater protection of sheep sperm from demethylation (Beaujean et al. 2004b). Why the zygote of some species requires rapid demethylation of the sperm pronucleus in the zygote is still an open question, but it may relate to the timing of embryonic genome activation, the composition of the genome, differences in reproductive strategies or it may be related to genomic imprinting (see Young & Beaujean 2004 for discussion). A more limited global demethylation event than in the mouse is also observed in the sheep, cow and rabbit genomes between the zygote and the 8-cell stages (Bourc’his et al. 2001b, Beaujean et al. 2004a, Shi et al. 2004), but these do not appear to be globally remethylated, at least prior to the blastocyst stage. In the ruminants, this cleavage stage demethylation does not appear limited to a single parental genome.

At least for the mouse, information on other germline-specific chromatin modifications is emerging. Concurrent with higher transcriptional activity in the larger male murine pronucleus, hyperacetylation of paternal pronuclear DNA has been observed relative to the female pronucleus and sperm/metaphase II oocytes (Adenot et al. 1997). Arney et al. (2002) have demonstrated that metaphase II oocytes chromosomes and female pronuclei have high levels of methylation on H3-K9. The paternal genome shows no methylated H3-K9 immunostaining shortly after fertilisation and it is suggested that this differential chromatin modification recruits maternally stored heterochromatin protein, Hp1β, exclusively to the maternal genome. This early recruitment of heterochromatin-associated proteins provides a plausible mechanism for masking maternal chromatin from demethylation at the pronuclear stage, a question that will perhaps be unravelled by interspecies studies.

**New experimental models for germline epigenetic investigation**

The difficulty in obtaining and analysing the small number of PGCs and their derivatives even in the mouse embryo has limited the studies on epigenetic reprogramming that have been carried out to date, despite this appearing to be a fundamental developmental event with severe consequences if misregulation occurs. Several experimental models have recently been developed which may lead to novel sources of material for study in the future, particularly in the human.

**EG cells**

In vitro transformation of mouse EG cell lines from PGCs (Matsui et al. 1992, Resnick et al. 1992) results in similar cell appearance, proliferation characteristics and marker expression (McLaren & Durcova-Hills 2001) but, unlike PGCs, EG cells are pluripotent (Matsui et al. 1992) and can form germline chimeras (Labosky et al. 1994a, Stewart et al. 1994). Mouse EG cells derived from PGCs isolated at different stages during development have shown that these cells largely undergo epigenetic modifications characteristic of the germline in vivo (Hajkova et al. 2002).

Imprint analysis on EG cells derived from 8.0–9.5 dpc PGCs revealed some degree of erasure (Labosky et al. 1994b, Stewart et al. 1994, Durcova-Hills et al. 2001), consistent with the nuclear transplantation studies of Yamazaki et al. 2003. Extensive hypomethylation of the...
Igf2r, Peg3, Peg1/Mes, p57Kip2 and Nnat imprinted genes has been observed in female and male EG cells derived from 11.5–12.5 dpc PGCs (Labosky et al. 1994b, Tada et al. 1998). Chimeras made with post-migratory EG cells show fetal overgrowth and skeletal abnormalities, consistent with the reduced methylation of paternally imprinted genes. Hypomethylation was more evident in EG cells with the reduced methylation of paternally imprinted genes. Hypomethylation was more evident in EG cells derived from male embryos (Tada et al. 1998), a feature dependent on the cellular sex chromosome constitution rather than on the sex of the genital ridge (Durcova-Hills et al. 2004). Of interest is that 12.5 dpc mouse EG cells can induce reprogramming of a somatic nucleus in cell hybrids by changing the methylation status of imprinted and non-imprinted genes (Tada et al. 1997). Many attempts to derive EG cell lines from later developmental stages have not been successful thus far.

Whether EG cells continue to follow a developmental programme that is already established for the PGCs in the embryo or whether there is a progressive loss of methylation during culture (Labosky et al. 1994a, b, McLaren 2003, Durcova-Hills et al. 2004) is open to question. The latter hypothesis is supported by the evidence that an altered methylation profile has been detected in mouse embryonic stem (ES) cells in culture (Dean et al. 1998).

More recently, EG cell lines have been derived by using human PGCs from gonadal ridges and mesenteries from 5–11 weeks post-fertilisation (Shambrott et al. 1998, 2001). The human EG cells resemble mouse EG cells in terms of phenotype, marker expression and pluripotent characteristics (Shambrott et al. 1998, 2001); however, the maintenance of these lines in culture has only been achieved up to 20 passages (Shambrott et al. 1998, Turnpenny et al. 2003), making their characterisation very difficult. The methylation profile of imprinted genes has thus far only been analysed in human EG cell-differentiated derivatives, because of the impossibility of maintaining the undifferentiated EG cells in culture (Onyango et al. 2002). In comparison with derivatives of mouse EG cells derived at 8.5 dpc, human EG cell derivatives from fetuses of 5–11 weeks post-fertilisation maintained the parental imprinting status, and the authors suggest that the time of imprint erasure differs in the two species. Since the human EG cell lines were reported to be derived from both the mesentery (en route to the gonadal ridge when mouse PGCs have only undergone limited demethylation) and from the gonadal ridge (where mouse PGCs undergo dramatic erasure after 1–2 days), further examination of individual lines and information on imprinting status before differentiation or the equivalent differentiated cell types in vivo is required to substantiate these conclusions.

**Germ cells from ES cells**

An exciting development in terms of potentially providing germ cell material for epigenetic study follows the observations that mouse and human ES cells can differentiate to germ cells and their male and female derivatives in vitro. Mouse ES cells can spontaneously differentiate to different stages of germ cell development expressing markers of the pre-migratory (oct4, cKit) and post-migratory (vasa; Hubner et al. 2003, Toyooka et al. 2003) phases. ES-derived germ cells were able to develop ovarian follicle homologues with oocytes that can enter meiosis and form parthenogenetic blastocyst-like structures (Hubner et al. 2003). However, the lack of expression of the zona pellucida 2P1 questions the ability of these oocytes to undergo normal fertilisation. Recent advances in promoting gametogenesis in vitro may combine with ES technology to improve the efficiency of mimicking full germline differentiation in vitro. Notably, mouse pre-meiotic female germ cells isolated from the 12.5 dpc fetus have recently been stimulated to undergo substantial oogenesis in vitro (Obata et al. 2002). The isolated oocytes at different stages of development showed the expected in vivo methylation pattern of the Igf2r imprinted gene, suggesting that epigenetic reprogramming is being accomplished in vitro. However, another study reported inappropriate methylation of the Igf2r and Peg1 loci during in vitro oocyte growth in pre-antral follicle culture ( Kerjean et al. 2003), thus factors such as optimisation of culture conditions will prove an important consideration in experimental design.

It has also been demonstrated that mouse ES cells can form post-migrating-type male germ cells in differentiating embryoid bodies (EBs) (Toyooka et al. 2003). This differentiation can be stimulated by BMP4-producing cells, mimicking the pathway for germ cell specification in vivo. These can undergo meiosis and spermatogenesis and differentiate into sperm when transplanted into the testis capsule. Geijsen et al. (2004) stimulated mouse EB-derived PGCs with retinoic acid to differentiate haploid round spermatids that could initiate fertilisation of oocytes after ICSI, suggesting that ES cells can give rise to germ cells by several routes in vitro. Interestingly, it has been shown that it is possible to derive EG cell lines from mouse ES-derived germ cells (Geijsen et al. 2004). These EG cells show a somatic-like imprinting status of the Igf2r and H19 genes when derived from day 4 EBs, but the imprinting methylation profile was erased at day 7 of EB differentiation, demonstrating that the PGCs derived from EBs may be able to mimic the epigenetic reprogramming features of PGCs developing in vivo.

To date, just one attempt to derive germ cells from human ES cells has been reported (Clark et al. 2004). Human ES cells were differentiated spontaneously through EB formation and were shown to give rise to cell-expressing germ cell markers. In contrast to the mouse system, human ES-differentiated germ cells expressed both female (GDF9, oocyte-specific) and male (TEKT1, spermatid-specific) germine markers. However, a low degree of meiotic marker expression was detected during human ES differentiation, suggesting that the spontaneous production of gametes in vitro is a more inefficient process in human. Cells with PGC or gametic phenotypes remain to be
isolated from human ES cells, but the likely possibility that this will be achieved will give rise to many exciting new experimental possibilities.

**Novel model organisms**

While lower model organisms have previously provided powerful tools for examining developmental mechanisms conserved in mammals, germline specification and epigenesis studies have been hampered by the use of germplasm, rather than epigenesis, to form PGCs in species such as Xenopus, zebrafish and Drosophila. Intriguingly, Johnson et al. (2003a,b) have recently demonstrated that the axolotl makes PGCs in a mechanism highly conserved with mammals, involving BMP4 induction, and the switching on of oct4, vasa and dazl. Since neither zebrafish or Xenopus undergo the genome-wide demethylation reprogramming event that mammals undergo post-fertilisation (MacLeod et al. 1999, Stancheva & Meehan 2000) it will be of interest to compare the scenario in axolotl, both post-fertilisation and during PGC formation.

**The future**

The above examples of continuous development of methods for the formation and survival of primordial germ cells and gametes in culture constantly provides new opportunities to study and modify the key developmental process of epigenetic reprogramming. It is very likely that procedures in both ART and somatic cell nuclear transfer could be improved and become more efficient and safer if a better understanding of epigenetic reprogramming in the germline and preimplantation embryo were available.

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