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GAMETOGENESIS '98
Cytosine Methylation and the Unequal Developmental Potentials of the Oocyte and Sperm Genomes

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Introduction

Reciprocal deficiencies in developmental potential are imposed on the oocyte and spermatozoan genomes during gametogenesis; normal development of offspring therefore demands an equal contribution of chromosomes from each parent. Genomic imprinting, the epigenetic change that marks a gamete's haploid genome as maternal or paternal, was seen first in uniparental mouse embryos made by pronuclear transplantation. Replacement of the male pronucleus of a zygote by a second female pronucleus gives rise to a gynogenote; replacement of a female pronucleus by a second male pronucleus forms an androgenote (McGrath and Solter 1984; Surani et al. 1984; reviewed by Solter 1988). Mendelian considerations would predict normal offspring (except in the case of YY androgenotes), but development is abortive in all cases. Gynogenotes show severe deficiencies in extraembryonic structures but a relatively normal embryo, whereas development of the embryo is affected more severely than extraembryonic structures in androgenotes. Nonetheless, both androgenotes and gynogenotes die prior to midgestation.

Uniparental conceptuses are quite common in humans, and their development is comparable to that of uniparental mouse embryos (reviewed by Mutter 1997). Androgenetic conceptuses present as complete hydatidiform moles and result from loss of the maternal chromosomes soon after fertilization. These moles contain only paternal chromosomes and no detectable embryonic tissues; they are composed of masses of hydropic chorionic villi and other placental structures. Dermoid cysts that contain only maternal chromosomes arise from parthenogenetically activated oocytes. These develop into a mass of well-differentiated but highly disorganized adult tissues that often includes tooth, bone, cartilage, skin, and other tissues. Extraembryonic structures are usually absent.

Uniparental disomy of single chromosomes or chromosomal regions produces characteristic effects if the affected regions contain imprinted loci. Such regions have been mapped in systematic studies of mice that bear Robertsonian translocations (reviewed by Solter 1988). A number of human genetic disorders and cancers have been shown to involve uniparental disomy or loss of heterozygosity (LOH) in favor of one parental allele at imprinted loci (reviewed by Tycko, in press). In the best-studied example, uniparental inheritance of human 15q causes Prader-Willi syndrome when the maternal chromosome is duplicated, whereas duplication of the paternal chromosome causes the unrelated Angelman syndrome. Beckwith-Wiedemann syndrome can be caused either by uniparental inheritance of 11p such that paternal alleles are duplicated or by LOH in favor of paternal alleles. Some tumor-suppressor genes are also subject to genomic imprinting, as shown by strong biases against alleles of a specific parental origin in cases of LOH (Tycko, in press). More than 20 imprinted genes have now been identified, and the parental origin of the expressed alleles has been confirmed by direct assays. Imprinted genes play diverse roles and have no obvious common function (Beechey, in press).

Genomic Imprinting and Cytosine Methylation

Genomic imprinting requires that two identical or nearly identical DNA sequences show highly unequal levels of expression over long periods of time. This is something of a biochemical embarrassment, since the inactive allele is clearly in the presence of all the factors required for its transcription, as proved by the activity of the other (identical) allele in the same nucleus. Equilibration of transcription factors between identical alleles should occur with time, and any inequalities of transcription should cease, but most imprinted genes maintain stable allelic differences for long periods.

The answer seems to lie in a heritable chemical dif-

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ference between the alleles, in the form of methyl groups at the 5 position of cytosine residues. Most 5-methylcytosine is within the self-complementary dinucleotide CpG, and many (but not all) imprinted loci have been shown to bear allele-specific methylation patterns in the vicinity of transcription start sites (Tycko 1997). Methylation of promoter regions has been shown to repress transcription via proteins that recognize methylated sequences; these proteins recruit transcriptional repressors and trigger assembly of the methylated sequences into the condensed state (Tate and Bird 1993; Kass et al. 1997). Methylation patterns are subject to clonal inheritance via preferential methylation of hemimethylated sites, which are produced by semiconservative DNA replication. The heritability of methylation patterns could underlie the remarkable stability of allele-specific gene expression in genomic imprinting.

Both the heritability of methylation patterns and their strong suppressive effects on transcription indicate that allele-specific methylation might be involved in allele-specific gene expression; the importance of that role can be inferred from the following lines of evidence. First, a number of imprinted genes show allele-specific methylation patterns that are established during gametogenesis and are maintained through to adulthood. Second, several imprinted genes have been shown to switch to a biallelic pattern of expression in mouse embryos deficient in Dnmt1, the major DNA methyltransferase of mammals (Li et al. 1993). Third, it is striking that genomic imprinting of the sort observed in mammals has not been reported in organisms whose DNA is not covalently modified; gene silencing at nonmutant loci in such species does not show a reproducible allele-specific component. Fourth, somatic loss of allele-specific expression can involve the development of biallelic methylation patterns, which appear to result from transfer of methylation patterns from the more-methylated allele to the less-methylated allele (Bestor and Tycko 1996). Although the possible involvement of other chromatin-mediated mechanisms must be examined, the evidence for an essential role of allele-specific cytosine methylation in genomic imprinting is compelling.

Erasure of Epigenetic Information in Primordial Germ Cells

Little is known of the mechanisms by which specific sequences are designated for de novo methylation in the germ line or somatic tissues. Although much remains to be learned, some recent findings have identified specific developmental windows during which demethylation and de novo methylation might create allele-specific methylation patterns at imprinted loci.

Imprinted genes must be capable of switching between maternal and paternal expression potential in each reproductive cycle; this requires the removal of preexisting methylation patterns in the germ line. It has been known for some time that primordial germ cells have severely demethylated genomes (Monk et al. 1987; Driscol and Migeon 1990). Surani and his colleagues (Tada et al. 1997) found that fusion of somatic cells to embryonal germ cells (totipotent permanent cell lines derived from primordial germ cells) leads to the sweeping demethylation of the somatic-cell chromosomes when assayed after many cell doublings in culture (Tada et al. 1997). Allele-specific methylation at the imprinted *Peg1/Mest* gene was erased, as was allele-specific transcription. There was also reactivation of the inactive X chromosome derived from the somatic cell–fusion partner. Demethylation is therefore a dominant property of primordial germ cells.

Is demethylation active (mediated by factors that directly remove methylated bases or methyl moieties) or passive (dependent on DNA replication in the absence of maintenance methylation)? Several laboratories have reported biochemical evidence of very unusual "demethylases" in extracts of somatic cells (Weiss et al. 1996; Jost et al. 1997), but no gene for a mammalian demethylase has been identified, and no data have yet appeared that bear on a role for such an enzyme in the shaping of methylation patterns. Arguing against an active mechanism are the recent data of Matsuo et al. (1998), who found that demethylation in *Xenopus* embryos has a requirement for both replication of the methylated DNA and the binding of transcription factors near the methylated site. The replication dependence strongly suggests that passive demethylation operates in this system. However, the possibility of active demethylation cannot be excluded by the available data.

De Novo and Maintenance Methylation in the Germ Line

It was predicted >20 years ago that de novo and maintenance methylation would be mediated by two distinct groups of enzymes: (1) de novo enzymes produced at particular stages of gametogenesis and early development, which would methylate specific sequences, and (2) sequence-independent maintenance enzymes that could only perpetuate patterns created by the de novo enzymes. These predictions seem to have been widely accepted as fact. Consistent with this model, the predominant DNA methyltransferase in mammalian cells, Dnmt1, concentrates at replication foci in S-phase nuclei (Leonhardt et al. 1992) and preferentially methylates hemimethylated DNA (Yoder et al. 1997*a*). However, Dnmt1 has substantial de novo activity and is the predominant de novo activity in every tissue and cell type examined to date (Yoder et al. 1997*a*). Furthermore, Dnmt1 is present at high levels in postmitotic germ cells, in which there is no maintenance methylation but in which substantial de novo methylation occurs (Trasler et al. 1990). Genetic

data require that there be at least one additional DNA methyltransferase that may have a specialized role in the silencing of newly integrated retroviral DNA (Lei et al. 1996), but there is little evidence of a family of sequence-specific DNA methyltransferases active during gametogenesis. The evidence suggests that Dnmt1 is the major DNA methyltransferase in both de novo and maintenance methylation and that its de novo activity is controlled by other factors (Yoder et al. 1997*a*).

Regulation of Dnmt1 by Alternative Splicing and Protein Sequestration in Postmitotic Germ Cells

Dnmt1 protein is present at high levels in postmitotic spermatocytes and is segregated into distinct nuclear domains during an interval of the leptotene/zygotene stages (Jue et al. 1995). These nuclear domains may be the site of de novo methylation of imprinted loci, and the leptotene/zygotene stages may therefore include the point at which de novo methylation occurs at paternally imprinted loci. Dnmt1 protein abruptly disappears at the pachytene stage, although the mRNA remains abundant. At this stage a pachytene spermatocyte-specific 5′ exon is found on nearly all of the Dnmt1 mRNA population (Mertineit et al. 1998). This exon has multiple short open reading frames that interfere with translation, and the pachytene-specific mRNA is not associated with polyribosomes. We have suggested that recombination intermediates are vulnerable to dysregulated de novo methylation (Bestor and Tycko 1996) and that the post-transcriptional down-regulation of Dnmt1 production at the pachytene stage of spermatogenesis may serve to protect meiotic chromosomes against de novo methylation.

Postmitotic oocytes contain very large amounts of Dnmt1 protein, which accumulates during the postnatal oocyte growth phase. However, this protein is sequestered in the cytoplasm and is localized to the nucleus only in early growing oocytes (Mertineit et al. 1998). Nuclear-transplantation studies have shown that oocyte nuclei lose gynogenetic developmental potential and gain biparental developmental potential during oocyte growth (Kono et al. 1996), and it has been suggested that Dnmt1 participates in de novo methylation of maternally imprinted genes during the oocyte growth phase (Mertineit et al. 1998). Mature oocytes contain very large amounts of Dnmt1 protein (~30,000-fold more than a cycling somatic cell; Carlson et al. 1992). However, this protein is encoded by an mRNA that contains a 5′ exon different from the 5′ exon present in either the pachytene spermatocyte-specific mRNA or the mRNA found in all cycling somatic cells. The oocyte-specific mRNA encodes a protein that lacks 113 N-terminal amino acids; this truncated protein is assembled into a shell just within the cortex of the ovulated oocyte. The truncated oocyte-specific protein bears a functional nuclear-localization sequence and must be actively retained in the cytoplasm; it is localized to nuclei at the eight-cell stage and at all stages of postimplantation development. The very large maternal store of Dnmt1 protein is enzymatically active and is sufficient to support development of homozygous *Dnmt1*-null embryos to day 8.5 of gestation (Carlson et al. 1992; Li et al. 1993).

The cytological data suggest that paternally imprinted genes may undergo de novo methylation during the leptotene/zygotene stage of spermatogenesis (prior to meiotic recombination), whereas maternally imprinted genes may undergo de novo methylation in the growing oocyte (after meiotic recombination). These predictions can now be tested directly by evaluation of methylation changes in DNA from purified populations of germ cells.

What Features of Imprinted Genes Cue De Novo Methylation during Gametogenesis?

A set of defined sequences that reliably confer maternal or paternal imprinting on a mouse transgene have yet to be identified, and the nature of the *cis* elements that distinguish imprinted genes is not understood. However, a number of features have been suggested to be characteristic of imprinted genes. Such genes tend to be rich in short direct repeats (Neumann et al. 1994), and some appear to have arisen by retrotransposition of transcripts of nonimprinted genes (Nabetani et al. 1997). Imprinted genes often have few introns, and these tend to be of small size (Hurst et al. 1996). At least three imprinted loci (*H19, Ipw,* and an antisense transcription unit within the imprinted *Igf2r* gene) encode a nontranslated RNA of unknown function (reviewed by Beechey, in press). Some imprinted loci are clustered; both the imprinted region involved in the Prader-Willi/Angelman syndromes and that involved in the Beckwith-Wiedemann syndrome contain multiple imprinted genes (Tycko 1998). There is also evidence for interaction of imprinted loci; in situ hybridization of interphase nuclei indicates that the maternal and paternal alleles of the Prader-Willi/Angelman imprinted domain approach each other at a point late in S phase (LaSalle and LaLande 1996), and the expression and methylation of the endogenous *U2af1-rs1* and *Igf2* (two imprinted genes in mice) are affected when homologous copies are present as transgenes (Hatada et al. 1997; Sun et al. 1997).

The principal function of cytosine methylation in eukaryotes is defense against proliferation of parasitic sequences (Bestor 1990; Yoder et al. 1997*b*), and the large majority of the 5-methylcytosine in the mammalian genome lies within parasitic sequence elements (retroposons and endogenous retroviruses), which represent $\geqslant 35\%$ of the genome and are rich in methylated CpG dinucleotides (Yoder et al. 1997*b*). Barlow (1993) has suggested both that imprinted genes display cues that

resemble those used by the cell to identify parasitic sequences and that methylation imprinting is the result of the different efficiencies with which those cues trigger de novo methylation in the male versus female germ lines (Bestor and Tycko 1996).

Genomic Imprinting and the Cloning of Mammals

Nuclear-transplantation studies in mice have shown that biparental diploid nuclei lose totipotency very rapidly during preimplantation development; a nucleus from a cell of the blastocyst inner-cell mass cannot support development of an enucleated zygote (reviewed by Solter 1988). This developmental restriction may be related to postzygotic changes in imprinting status, as some imprinted genes, including *Igf2*, undergo tissue-specific changes in allele-specific expression during normal embryogenesis (Efstratiadis 1994). With age, cytosine methylation also encroaches on the promoters of genes (Ono et al. 1993); and, also with age, ectopic methylation of imprinted genes has been reported (Issa et al. 1996). These somatic changes in epigenetic states had seemed to obviate the cloning of adult mammals (McGrath and Solter 1984); the report of a sheep cloned from an adult somatic nucleus (Wilmut et al. 1997) was therefore surprising (and has been the subject of recent controversy; see Sgaramella and Zinder 1998). It is quite possible that failure to reset methylation patterns and epigenetic states (as normally occurs during gametogenesis) may affect the development of clonal offspring derived from transplantation of nuclei from differentiated cells into enucleated zygotes. Even if it becomes possible to clone animals with fair efficiency, genomic imprinting and other epigenetic effects might give rise to substantial and unpredictable phenotypic variation among clonal offspring.

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