CHAPTER 6

Epigenetic modification during oocyte growth and maturation

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INTRODUCTION

The growth and maturation of the oocyte is a complex and highly regulated process. An important aspect of oocyte maturation is the establishment of the correct epigenetic status. 'Epigenetics' refers to processes such as DNA methylation or histone modifications that regulate gene activity without affecting the actual DNA sequence, but are heritable through cell division. The epigenetic state of the male and female germ cells is not equivalent; this was first discovered as a result of ingenious nuclear transplantation experiments carried out in the mid 1980s demonstrating that uniparental embryos are not viable[1,2]. Subsequently, a subset of mammalian genes was found to be subject to genomic imprinting. Importantly, a number of imprinted genes are essential for fetal growth and development, including the functioning of the placenta. These genes are expressed in a parent-of-origin specific manner, as a result of the different epigenetic profiles acquired by imprinted genes during male and female gametogenesis. The best characterized epigenetic modification is the methylation of cytosine residues in DNA, which is involved in establishing genomic imprints in the germ line. This establishment occurs during the growth phase of oocyte development, and is beginning to be elucidated in greater detail. In recent years, increased concern has been focused on the potential for epigenetic dysregulation as a result of early embryo culture and assisted reproductive technologies[3,4].

Several recent reports have suggested that there might be an increased occurrence of the imprinting disorders Beckwith–Weidemann syndrome[5–8] and Angelman syndrome[9,10] in children conceived by in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Of particular interest is the fact that the described cases are almost all the result of maternal DNA methylation defects. These cases highlight the need for further study of the possible mechanisms of epigenetic dysregulation during assisted reproduction. In-vitro maturation and IVF raise concerns due to the prolonged exposure of the oocyte and early embryo to culture conditions and the use of exogenous gonadotropins. However, there may also be epigenetic causes for infertility in patients undergoing assisted reproductive technologies (ARTs). This chapter will review our current knowledge of the epigenetic modifications that occur in developing female germ cells including the erasure and establishment of methylation imprints, the aspects of current

ARTs, and the conditions they are designed to treat (i.e. infertility) that may perturb or reveal imprinting defects and suggest further studies that are required in this area.

DYNAMICS AND TIMING OF EPIGENETIC CHANGES IN THE OOCYTE

The oocyte undergoes many well-defined epigenetic changes during its growth and maturation. Levels of DNA methylation decrease in the primordial germ cells, which will give rise to the oocytes, as they migrate into the genital ridge. Following this period, methylation of a class of genes, referred to as imprinted genes, must be acquired in order to direct the expression of these genes from a single allele. The functional non-equivalence of the male and female germ lines was first discovered based on the results of nuclear transfer experiments in which uniparental embryos failed to develop normally[1,2]. Androgenetic mouse embryos created by the combination of two male pronuclei, hence lacking any maternal contribution, give rise to well-developed extraembryonic tissues with poor embryonic development. In contrast, gynogenetic embryos (containing two maternal pronuclei and no paternal contribution) or parthenogenetic mouse embryos derived by the activation of oocytes develop, at least for a short period, as relatively normal embryos, but have rather poor development of the extraembryonic tissues. Based on these studies, it was proposed that genes expressed from the paternal genome direct development of the extraembryonic tissues, in order to ensure optimal nutrient exchange in support of the developing embryo. In contrast, the maternal genome expresses genes that are involved in the development of the embryo proper. These observations led to the well-known 'conflict hypothesis' of genomic imprinting[11,12]. This hypothesis proposes that the paternal genome evolved in such a way that genes are expressed to favor the optimal use of maternal resources in order to maximize fetal growth and development, while the maternal genome attempts to limit the investment in fetal growth to reserve resources for future pregnancies. Since the initial studies involving uniparental embryos, a subset of genes has been discovered that is expressed from a single allele only, depending on whether the gene is inherited on the maternally or paternally derived chromosome. The phenomenon leading to this uniparental expression is known as genomic imprinting.

Genomic imprinting is controlled by epigenetic means, as DNA sequence alone cannot distinguish between parental alleles or control allele-specific expression of genes. DNA methylation plays an important role in genomic imprinting, through the differential methylation of the parental alleles. Genomic imprinting is also associated with histone modifications, antisense transcripts, and non-coding RNAs, although the mechanisms are not well understood. As such, this chapter will concentrate on the role of DNA methylation in the oocyte.

Erasure of DNA methylation imprints in the mouse model system

The germ line arises from the migration of primordial germ cells into the genital ridge. The primordial germ cells appear to be marked by normal somatic DNA methylation patterns when they begin their migration, however these cells undergo widespread DNA demethylation around embryonic day (E) 10.5, as they migrate into the genital ridge. The earliest studies examining DNA methylation in the germ line used methylation-sensitive restriction enzymes together with Southern blotting or polymerase chain reaction (PCR). The results of these studies suggested that the primordial germ cell genome is completely demethylated by E13.5[13–16]. More recently, bisulfite sequencing has been used to further characterize the methylation sta-

tus at various stages (Figure 6.1a). The methylation status of several imprinted genes has been examined, these include *H19, Snrpn, Peg3, Kcnq1ot1* (also named *Lit1*), *Igf2, Gtl2*, and *Rasgfr1*, as well as non-imprinted gene sequences such as α-*actin* and *my1C*[17,18]. These studies indicated that imprinted and non-imprinted single copy genes become completely demethylated between E10.5 and E13.5. Intriguingly, similar studies examining the methylation status of some repeat sequences, including long interspersed nuclear element 1 (LINE1), intracisternal A particle (IAP), and minor satellite sequences, found that these repetitive sequences are only partially demethylated in the primordial germ cell population[17,19–21] (Figure 6.2).

Analysis of gene expression has also been undertaken to examine the progress of epigenetic reprogramming in the germ line (Figure 6.1b). In primordial germ cells, monoallelic expression of imprinted genes would be expected prior to the erasure of methylation imprints. At E9.5, monoallelic expression of four imprinted genes was detected[19]. As development progressed, biallelic expression of *Snrpn* (E10.5) and of *H19* and *Igf2* (E11.5) was detected. Analysis of embryos generated by somatic cell nuclear transfer using nuclei from primordial germ cells isolated from mice at various gestational ages has also pointed to demethylation of the genome in primordial germ cells between E10.5 and E12.5[22,23].

Taken together, these results indicate that single copy genes, both imprinted and non-imprinted, undergo a rapid, and perhaps active, demethylation as the primordial germ cells migrate into the genital ridge. Repetitive sequences do not appear to be subject to the same complete demethylation process, but do undergo a partial demethylation during the same period. As a result of DNA demethylation, evidence to date suggests that the primordial germ cells of both sexes are epigenetically equivalent by E13.5[17,18,22,24].

Maternal imprint establishment

Initial studies in mice examining a few CpG sites in the endogenous imprinted gene *Igf2r*[25–27] and the imprinted transgenes *RSVIgmyc* and *MPA434*[14,28] first suggested that maternal imprints are acquired during oocyte growth. Further support for the functional importance of DNA methylation occurring during oocyte growth came from nuclear transplantation studies in which parthenogenetic embryos containing one genome from a neonate-derived non-growing oocyte and the other genome from a fully grown oocyte developed to E13.5, 3 days longer than normal parthenogenotes (in which both genomes were derived from fully grown oocytes,[27] Figure 6.3b). In these experiments, immature, non-growing oocytes were collected from mice at postnatal day 1, and these oocytes were fused to enucleated germinal vesicle (GV)-stage oocytes in order to provide the correct cytoplasmic environment for the fused oocytes to proceed to the metaphase (MII) stage. The chromosomes of the fused oocytes were then transferred to mature ovulated MII stage oocytes (whose nuclear material was removed) and the resulting parthenogenetic embryos were allowed to develop in pseudopregnant recipient mice[27,29] (Figure 6.3b). These embryos contained only one set of maternally imprinted chromosomes from the fully grown MII oocyte, and one set of chromosomes without any maternal imprint, from the non-growing oocyte. Expression studies in the resulting parthenogenetic embryos, made possible due to single nucleotide polymorphisms between the strains used (Figure 6.1b), confirmed that the paternally expressed genes *Snrpn*, *Peg1*, and *Peg3*, which are not normally expressed from the maternal genome, were expressed from the genome derived from the non-growing oocyte[29], whereas they were not expressed from the genome of the fully grown oocyte. This result suggests that primary maternal imprints are not yet established in immature non-growing oocytes.

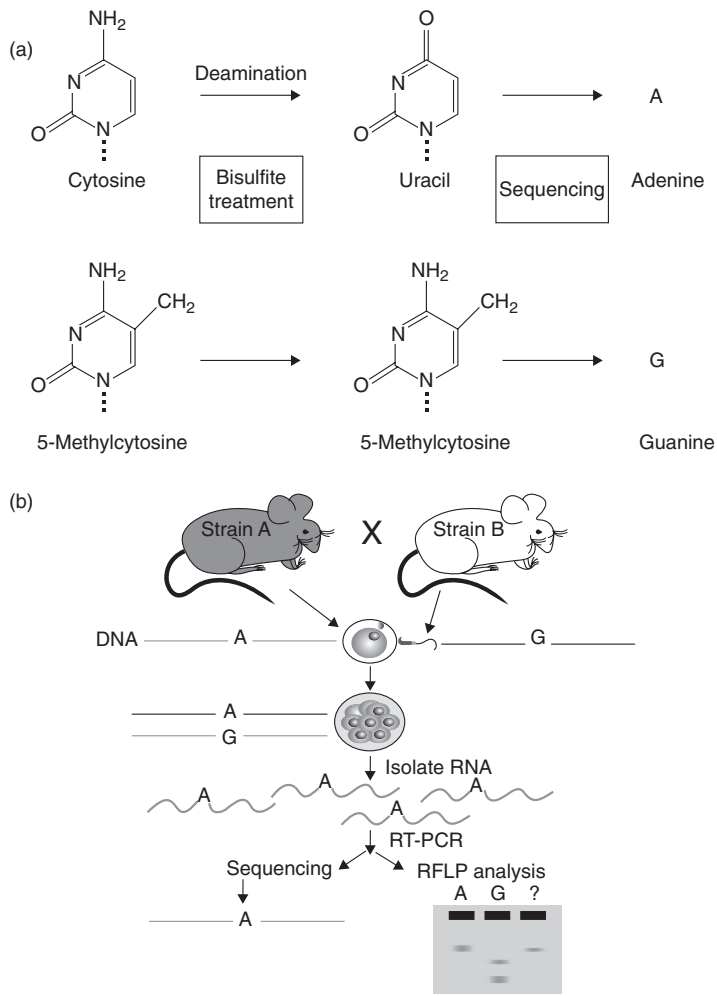

Figure 6.1 Schematic representation of techniques used to assess CpG methylation and allele-specific expression of imprinted genes in the mouse model. (a) Principle of bisulfite sequencing. DNA is treated with sodium bisulfite, resulting in a deamination of all non-methylated cytosine residues to uracil. 5-Methylcytosine residues are not modified in this reaction. Sequencing of the bisulfite-treated DNA allows for the identification of methylated and unmethylated cytosines within the region under study. (b) The presence of single nucleotide polymorphisms (SNPs) allows for identification of alleles that are transcribed. Two different strains are mated, with known sequence variants at imprinted genes. RNA is collected from the resulting embryo, and gene-specific RT-PCR is carried out. The resulting cDNA product can be sequenced to identify the relative proportion of the parental alleles present. Alternatively, if the SNP generates or abolishes a recognition site for a restriction enzyme, the relative contribution of parental alleles may be assessed using restriction enzyme digestion followed by electrophoretic separation. RT-PCR: reverse transcription–polymerase chain reaction; RFLP: restriction fragment length polymorphism; ?: test sample

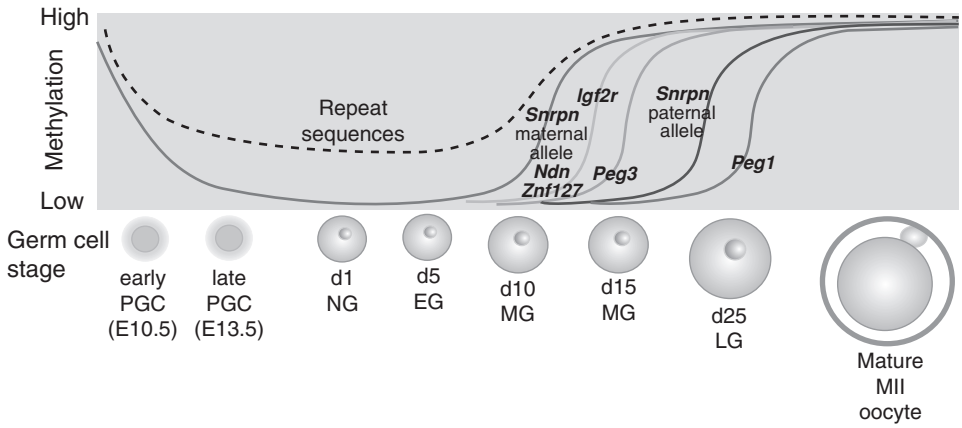

Figure 6.2 Methylation dynamics of imprinted genes and repeat sequences during female germ line development. The lower panel illustrates the stages of germ cell development shown in the top panel. The top panel illustrates the methylation dynamics of imprinted genes in the developing oocyte, beginning with the demethylation of imprinted and single copy genes in the primordial germ cells (red line). At this early stage, repeat sequences are not demethylated to the same extent as the single copy genes (dotted line). During oocyte growth, single copy genes become hypermethylated throughout the growth stages. Imprints are established asynchronously in the oocyte, with the latest imprints being established in the late growing stages. PGC: primordial germ cell; NG: non-growing oocyte; EG: early growing oocyte; MG: mid growth oocyte; LG: late growing oocyte; MII: metaphase II. Adapted from references 4 and 32 (see color plate section)

However, the resulting parthenogenotes could not develop to term, likely due to the absence of paternal imprints, since the non-growing oocyte genome would, along with the genome from the fully grown oocyte, express those genes that are normally maternally expressed (paternally imprinted), resulting in a double dose of these genes. This possibility was supported by expression analysis that revealed expression of *H19* and coordinate repression of *Igf2*, which shares a differentially methylated domain with *H19*, from the non-growing oocyte genome[29]. The result would be biallelic expression of *H19* and loss of *Igf2* expression in the parthenogenotes. This led the authors to repeat this experiment with non-growing oocytes from mice carrying a large deletion of the *H19* gene as well as the differentially methylated domain between *H19* and *Igf2*, which were then used for nuclear transfer to mature MII oocytes. As a result, two live born parthenogenetic pups were obtained, with a marked normalization in expression of most imprinted genes examined[30]. One of these pups was allowed to develop to adulthood, and even went on to produce offspring. This experiment demonstrated that limited parthenogenetic development is possible in the mouse provided that imprinted gene expression is appropriately controlled. This was, however, only possible with large deletions of an imprinted gene region. It is important to note that this experiment had a very low success rate, as 371 morulae were transferred to pseudopregnant females, but only two pups survived to birth. Clearly, many questions remain to be answered about parthenogenesis and the relative importance of imprinted genes in normal development.

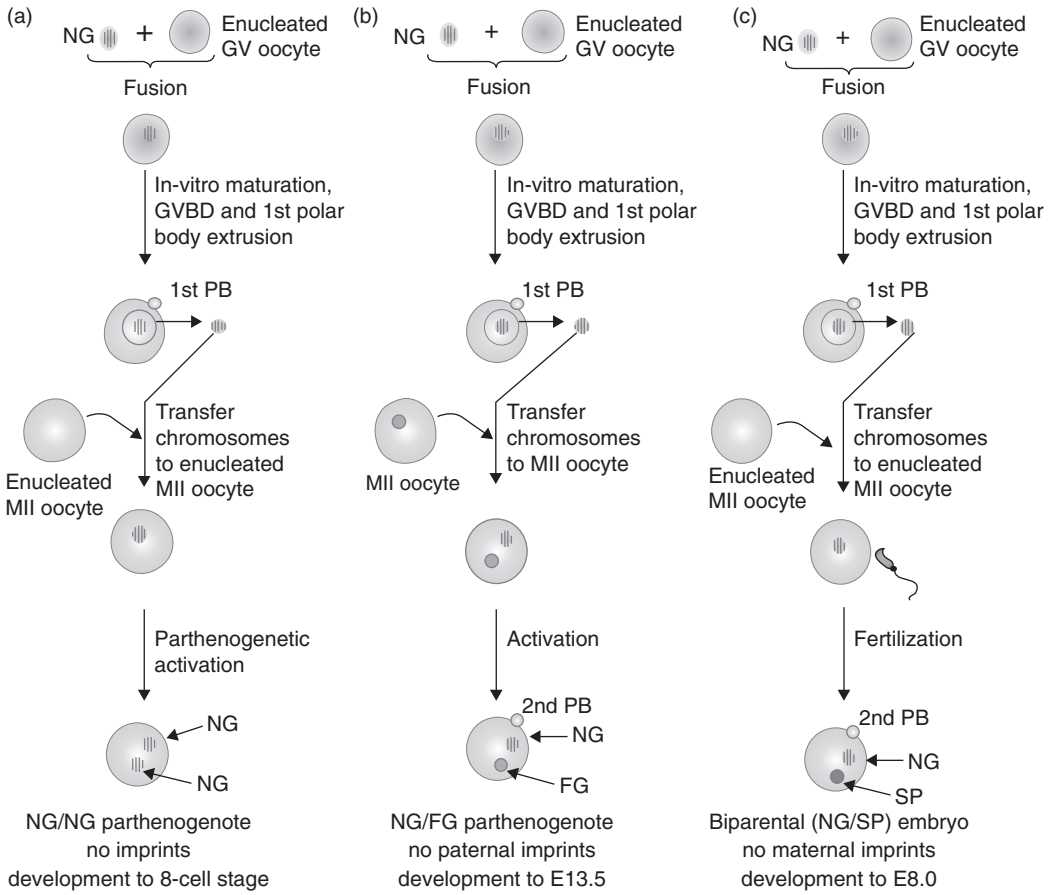

Figure 6.3 Schematic representation of nuclear transfer experiments revealing the developmental potential of oocytes at different stages of growth and maturation. Non-growing oocytes have not established maternal imprints (pink hatched nuclei). (a) When non-growing oocytes are used in nuclear transfer experiments followed by parthenogenetic activation of the oocyte, development stalls at the 8-cell stage. (b) When the nucleus of a non-growing oocyte is transferred to a mature MII oocyte which retains its nucleus (red nucleus) development proceeds to E13.5. (c) When the nucleus of a non-growing oocyte is transferred to an enucleated MII oocyte and fertilized with a normal spermatozoon (blue nucleus) development stalls at E8.0. NG: non-growing oocyte; GV: germinal vesicle stage oocyte; PB: polar body; MII: metaphase II; FG: fully grown (mature) oocyte; SP: sperm. Adapted from references 27 and 29 (see color plate section)

In other nuclear transplantation experiments, when the genomes from postnatal day 1 non-growing oocytes were transferred to enucleated MII oocytes, followed by parthenogenetic activation (ng/ng parthenogenetic embryos), development stalled at the eight-cell stage[27] (Figure 6.3a). In these parthenogenotes, no methylation imprints were present at all. In the absence of any methylation imprints, embryonic development cannot proceed to the blastocyst stage,

possibly due to problems in early cell specification. When non-growing oocytes were transferred to enucleated MII oocytes and fertilized in vitro (ng/sp embryos), development proceeded to about E8.0 (Figure 6.3c). In this case, paternal methylation imprints would be present, whereas maternal imprints would not. These embryos are not equivalent to androgenotes, as they only have one paternally imprinted set of chromosomes. Development of these embryos to E8.0 suggests that maternal imprints are required for postimplantation development, and there is some evidence that cell type specification is disrupted as early as the blastocyst stage[27].

The nuclear transplantation experiments established that maternal methylation imprints are not present in early non-growing oocytes, but are present in mature MII oocytes. In order to determine the time period during which these methylation imprints are established, embryos were generated by nuclear transfer using the genomes from oocytes isolated at different days of postnatal development paired with genomes from fully grown oocytes. These embryos were used to examine maternal imprint establishment by detection of transcripts that are normally paternally expressed[31]. Loss of expression of the paternally expressed genes examined was interpreted as indicating the establishment of the maternal methylation imprint. Embryos constructed with the nuclei of the earliest non-growing oocytes, isolated from postnatal day 1 ovaries, expressed all of the genes that are normally only expressed from the paternal genome[31]. Embryos constructed with postnatal day 5 oocyte genomes were beginning to exhibit loss of expression of *Snrpn, Znf127*, and *Ndn*, whereas those constructed with genomes from oocytes from postnatal day 10 ovaries began to exhibit loss of *Peg3* expression, while *Snrpn, Znf127*, and *Ndn* expression was completely lost in most of the samples. When genomes isolated from oocytes in mid-growth (postnatal day 15) were used, loss of *Peg1* expression was observed in some embryos, and once oocytes that had reached the fully grown stage in postnatal day 20 mice were used, imprints had been established at all paternally expressed genes in the resulting embryos[31] (Figure 6.2).

At the molecular level, bisulfite sequencing has been used to characterize the acquisition of DNA methylation imprints in oocytes at different stages of oocyte growth[32,33]. For these experiments, oocytes of increasingly larger sizes were isolated from ovaries collected at postnatal days 1, 5, 10, 15, and 25, similar to the experiments described above. Bisulfite sequencing (Figure 6.1a) was used to examine the methylation status of differentially methylated regions (DMRs) of four paternally expressed genes, *Snrpn, Igf2r, Peg1*, and *Peg3*. The results of this study indicated that imprint establishment occurred in a gene-specific manner, with *Snrpn* acquiring methylation first, followed by *Igf2r* and *Peg3*, while *Peg1* acquired the methylation imprint very rapidly in the latest stages of oocyte growth[32] (Figure 6.2). Importantly, this study also reports that the acquisition of the methylation imprint at the *Snrpn* DMR is related to oocyte diameter rather than specifically to the age of the female mouse. Oocytes isolated from postnatal day 15 ovaries were sized and the methylation status of the *Snrpn* DMR was assessed by bisulfite sequencing. In oocytes with diameters of 20–50 μm *Snrpn* was largely unmethylated, while in oocytes with diameters of 60–80 μm *Snrpn* was largely methylated[32].

Taken together, the nuclear transfer and bisulfite sequencing studies reveal that DNA methylation imprints are acquired progressively during the entire oocyte growth phase, as follicles progress from the primary to the antral stage. Interestingly, the methylation of specific genes is established at different stages in oocyte growth, with methylation of the genes examined being completed by the time oocytes arrest in MII (Figure 6.2). The difference in timing of imprint acquisition may be related to the different chromosomal locations of the imprinted gene clusters[32]. The relationship between oocyte size and methylation imprints could indicate that

imprint establishment requires the accumulation of proteins involved in the enzymatic process; in support of this hypothesis, the expression of the DNA methyltransferase genes *Dnmt3a, Dnmt3b*, and *Dnmt3L* peaked in oocytes from postnatal day 15 ovaries[32].

Additionally, it was found that for at least one locus, the *Snrpn* gene, the methylation imprint is first established on the maternal allele, and is only acquired later on the paternal allele[32]. This indicates that the alleles are not equivalent following erasure (Figure 6.2). It is perhaps methylation at other sites that were not examined or differences in chromatin structure that mark the different parental alleles, and then direct the establishment of methylation imprints[32]. Further studies will be required to ascertain if all methylation imprints are established preferentially on one allele before the other, and if chromatin structure plays a role in maintaining the identity of the alleles in the absence of methylation.

Given that methylation imprints appear to be established asynchronously, with certain imprints not being established until late in oocyte growth, it is possible that certain methylation imprints are more susceptible to perturbation as a result of ARTs. The susceptibility of different loci to disruption will also require further study.

Studies in humans and other animals

Due to ethical limitations, little has been done to examine imprint establishment in human oocytes. It is known that parthenogenetic embryos do not develop in vivo, but are the cause of ovarian teratomas[34]. The establishment of a single maternal methylation imprint at the human *SNRPN* DMR has been examined using bisulfite sequencing. In an early study, using unfertilized oocytes from a fertility center, the *SNRPN* DMR was found to be largely unmethylated in aspirated oocytes[35], leading the authors to suggest that the *SNRPN* methylation imprint is established after fertilization. A later study was conducted using GV-stage, metaphase I (MI) and MII oocytes that were unsuitable for transfer and were donated by patients for research purposes[36]. The *SNRPN* DMR was found to be highly methylated in GV stage oocytes, and the methylation was maintained in more mature oocytes. The results of this second study fit well with the data from the mouse, however more studies are required to clarify the timing of imprint establishment in human oocytes.

Hydatidiform moles are a common cause of gestational trophoblastic disease in women. Complete hydatidiform moles usually result from androgenetic pregnancies, and are marked by a complete lack of fetal tissue. An inherited form of complete hydatidiform molar pregnancy has been described, in which the resultant moles are phenotypically indistinguishable from the androgenetic complete hydatidiform moles, but on further genetic examination the moles are found to be biparental in origin[37]. Based on the recurrent, heritable incidence of biparental complete hydatidiform moles, as well as recurrence in women who have changed sexual partners, it was hypothesized that these biparental complete hydatidiform moles are the result of a defect in the maternal germ line[38]. Examination of the methylation status of several characterized imprinted gene DMRs was undertaken using bisulfite sequencing. Based on a limited number of clones, it appears that the maternal methylation imprint is absent in biparental complete hydatidiform moles[38]. These embryos are genomically equivalent to the ng/sp embryos generated by Kono et al.[27], where the genome of a non-growing oocyte lacking all methylation imprints was fertilized by a normal sperm nucleus. In the mouse, these embryos develop to approximately E8.0 with apparent developmental delay followed by embryonic loss[27]. Human biparental complete hydatidiform moles also fail to develop normally, with the human phenotype being much more severe than in the mouse, as there is an apparent lack of fetal tissue with expansive extraembryonic tissue[37].

The methylation status of four imprinted genes has been examined in tissues isolated from four different biparental complete hydatidiform moles, and the methylation patterns were found to be abnormal[39]. In particular, the genes that were normally maternally methylated showed a decrease in methylation and as a result were more like the paternal allele. Variability in the amount of hypomethylation was also observed between moles[39]. In contrast, the methylation status of imprinted genes was found to be normal in the somatic tissue of two women with recurrent biparental complete hydatidiform molar pregnancies[40], and thus the abnormal epigenotype of their molar pregnancies did not arise due to a general loss of methylation in all the mothers' cells. The studies conducted to date have not been able to identify whether the abnormal methylation pattern arose in the maternal germ line or after fertilization, as the moles studied were all from 6 weeks of gestation or later[40]. Judson et al.[38] have hypothesized that the underlying cause of familial recurrent biparental complete hydatidiform moles is a maternal germ line defect in which maternal imprints are not established. This is one possibility, although mutations in the known DNMT genes have been ruled out as causative in the familial biparental hydatidiform moles[41], implying that an as-of-yet unknown enzyme or protein is involved in the genesis of this disorder. El-Maarri et al.[40] proposed that the trophoblastic identity of the cells in complete hydatidiform moles may contribute to changes in methylation, as it has previously been shown that methylation is not strictly maintained in the placenta as it is in the embryonic compartment[42]. Clearly, more studies are required to determine the causation of familial recurrent biparental complete hydatidiform molar pregnancies.

The establishment of maternal imprints in oocytes has not been directly studied in other animal models. The majority of work has focused on techniques for parthenogenetic activation in vitro. Attempts have been made to generate parthenogenetic sheep, however these embryos die shortly after implantation and are growth retarded[43], while gynogenotes appear to develop normally at least to day 21 (implantation occurs between days 23 and 25 in sheep)[44]. However, later stages were not examined. Attempts have also been made to generate parthenogenetic marmoset monkeys[45]. These parthenogenotes developed to implantation but postimplantation development was limited.

THE DNA METHYLTRANSFERASES ARE INVOLVED IN ESTABLISHING METHYLATION IMPRINTS

As discussed above, methylation of important DNA sequences such as imprinted genes must be acquired during oogenesis to ensure proper gene expression in the embryo. The DNA methyltransferases (DNMTs) are currently the best-characterized enzymes involved in epigenetic reprogramming. The DNMTs catalyze a reaction in which a methyl group is transferred from the donor cofactor S-adenosylmethionine (SAM) to the 5′ carbon of a cytosine ring, resulting in 5-methylcytosine. In mammals, three families of DNMTs have been identified; these are grouped together based on sequence similarities in their C-terminal catalytic domains[46]. The DNMTs identified to date include DNMT1, which is the major DNMT in the mammalian system, as well as DNMT2[47], DNMT3a, DNMT3b[48], and DNMT3L[49,50]. The expression and activity of the DNMTs are summarized in Figure 6.4.

DNMT1

Mouse studies

DNMT1 has been assigned a role in maintenance methylation, based on early studies showing that this enzyme has a higher affinity for hemimethylated DNA than unmethylated DNA[51,52]. Three isoforms of *Dnmt1* transcripts have been

	PGC	NG	EG	MG	LG	MII
DNMT1	+	+	–	–	–	–
	maintenance methylation of repeat sequences					
DNMT1o	–	–	+	+	+	+
			cytoplasmic localization, no methylation activity			
DNMT3a	+	+	+	+	+	+
			de novo methylation – establishment of maternal imprints			
DNMT3b	+	+	+	+	+	+
			methylation of repeat sequences and chromosome stability			
DNMT3L	+	+	+	+	+	+
			de novo methylation – establishment of maternal imprints			

Figure 6.4 Summary of DNA methyltransferase (DNMT) expression and activity in the female germ line of the mouse. In the figure, (+) indicates that either transcript or protein expression has been detected at the stage indicated while (–) indicates that expression has not been detected to date. PGC: primordial germ cell; NG: non-growing oocyte; EG: early growing oocyte; MG: midgrowth oocyte; LG: late growing oocyte; MII: metaphase II oocyte

identified that use sex-specific first exons. *Dnmt1s* encodes the full-length protein that is expressed in somatic cells; *Dnmt1o* encodes an oocyte specific form of the protein, which lacks the first N-terminal 118 amino acids; while an untranslated isoform is generated in pachytene spermatocytes (*Dnmt1p*)[53,54]. During embryonic development, DNMT1 has been detected in primordial germ cells at E11.5 and remains strongly expressed until E13.5, at a time when methylation imprints are being erased, after which it decreases as cells enter meiotic prophase[17,55]. It has been suggested that during this stage of development, DNMT1 may be important for maintaining the methylation of repetitive elements, which do not undergo genome-wide demethylation to the same extent as imprinted genes. DNMT1o has been identified as the sole form of the protein that is present in the postnatal oocyte and the preimplantation embryo[54,56,57]. Detailed expression analysis revealed that DNMT1o is excluded from the oocyte nucleus after the early growing stage, and appears to be actively retained in the cortical region until the eight-cell stage postfertilization, when it enters the nucleus transiently, exiting by the 16-cell stage[54]. Based on this observation, it was postulated that oocytes must protect their meiotic chromosomes from inappropriate de novo methylation, raising the suggestion that DNMT1 may not act exclusively as a maintenance methyltransferase[54]. The ability of DNMT1 to act as a de novo methyltransferase in vivo remains to be determined.

To further examine the role of DNMT1o, the oocyte-specific isoform was deleted (knocked out) using gene targeting[56]. Male and female homozygous DNMT1o-deficient mice were obtained and appeared phenotypically normal, but the female mice were infertile. Characterization of this infertility revealed that the *Dnmt1o* knockout acts as a maternal effect lethal, where embryos derived from oocytes lacking DNMT1o rarely

survive to birth[56]. Analysis of global methylation in embryos from homozygous null female mice revealed that the genome was normally methylated, including repeat regions and single copy sequences. Also, analysis of methylation of the 5′ region of *Snrpn* in oocytes collected from DNMT1o-null females demonstrated that imprints were properly acquired in the oocyte. In contrast, analysis of the embryos derived from such oocytes uncovered a non-stochastic loss of methylation on half of the normally imprinted alleles[56]. Intriguingly, this occurred for genes that received their imprints from the maternal as well as from the paternal germ line, implicating a defect in maintenance methylation at one stage of preimplantation development, the eight-cell stage. Thus, methylation imprints were properly established in the absence of DNMT1o, ruling it out as the primary de novo DNMT responsible for establishing maternal imprints in the oocyte.

Studies in humans and other animals

The *DNMT1* gene is conserved among chordates (reviewed in Goll and Bestor[46]). *DNMT1o* transcripts have been detected in human[41,58] and opposum oocytes[59], but have not been detected in bovine oocytes[60]. To date, mutations of the *DNMT1* gene have not been associated with any human disorders.

DNMT3a and DNMT3b

Two functional DNMTs have been identified in the DNMT3 family. These enzymes are more closely related to DNMTs across many species[46,61]. Members of the DNMT3 family appear to act primarily as de novo DNMTs[48]. Based on gene targeting experiments, DNMT3a and DNMT3b also appear to have discrete functions[62].

Mouse studies

As mentioned earlier, *Dnmt3a* expression peaks in growing oocytes and coincides with the time of acquisition of maternal methylation[32]. Two different splice variants of *Dnmt3a* have been identified, *Dnmt3a* and *Dnmt3a2*[63]. *Dnmt3a* encodes the full-length protein that was initially characterized and is known to have methyltransferase activity[48], while *Dnmt3a2* encodes a form of the protein that lacks 219 amino acids at the N-terminus while retaining similar methylation activity in vitro[63]. In vivo, *Dnmt3a* transcripts are ubiquitously expressed, while *Dnmt3a2* transcripts are detected in a tissue-restricted manner, specifically in cells known to undergo de novo methylation such as testis, ovary, spleen, and thymus[64]. *Dnmt3a2* transcripts are detected at slightly higher levels than *Dnmt3a* transcripts in whole ovaries at postnatal day 12, a time period when waves of oocyte growth and de novo methylation are occurring[64]. Interestingly, DNMT3a2 has been shown to localize specifically to euchromatin while DNMT3a localizes to heterochromatin[63]. Based on these pieces of information, DNMT3a2 may be involved in de novo methylation of single copy sequences. When *Dnmt3a* is knocked out in mice, the homozygous null animals develop to term and appear normal at birth, however they die at about 4 weeks of age[62]. Methylation in the homozygous null animals appears to be normal[62], indicating that another protein must compensate or cooperate with DNMT3A in vivo. To determine if loss of *Dnmt3a* specifically in the germ line resulted in a reproductive phenotype, a conditional allele was generated[65]. When *Dnmt3a* was removed from the germ line only, the mice were viable and reached adulthood, but when the germ line *Dnmt3a*-null female mice were crossed to wild-type males, no live pups were obtained[65]. Further examination revealed an embryonic lethal phenotype by E10.5, which must be due to a maternal effect as the males would contribute a normal *Dnmt3a* gene to the embryos[65]. In addition, maternal methylation imprints were not established, and gene expression of imprinted genes was dysregulated in the E10.5 embryos derived from mutant mothers[65]. Thus, DNMT3a is one of the key

enzymes needed for the acquisition of maternal methylation imprints in oocytes.

One of the multiple isoforms of *Dnmt3b* likely can compensate for some of the functions of DNMT3a in its absence, as it has been shown that DNMT3a and DNMT3b have distinct functions in gene targeting experiments[62]. Six different isoforms of *Dnmt3b* have been identified to date, and all of the isoforms show different tissue-specific expression patterns[48,63,66,67]. *Dnmt3b1* and *Dnmt3b2* transcripts have been detected in the female germ line, however DNMT3b2 is the only functional isoform in the adult ovary and is therefore more likely to be important for de novo methylation in the oocyte[64]. *Dnmt3b2* and *Dnmt3b3* transcripts are both highly expressed in cells undergoing de novo methylation (testis, ovary, spleen, thymus, and liver), however DNMT3b3 appears to be unable to transfer methyl groups despite its ability to bind DNA[48,68,69]. As a result, it has been suggested that DNMT3b1 and DNMT3b2 may act as de novo methyltransferases while DNMT3b3 and possibly DNMT3b6 may be involved in regulating methylation[63]. *Dnmt3b* has been knocked out in mice, resulting in embryonic lethality[62]. Also, in homozygous null embryos recovered prior to E9.5, undermethylation of C-type retroviral sequences and IAP sequences was observed, suggesting that DNMT3b may be involved in the methylation of a subset of minor satellite repeats[62]. Again, to determine if loss of *Dnmt3b* specifically in the germ line caused a phenotype, a conditional allele was generated[65]. When *Dnmt3b* was removed from the germ line only, the mice were viable and reached adulthood, and live pups were obtained from matings of the DNMT3b-null females to wild-type males[65]. Methylation of the sequences examined was not affected in the offspring of mutant animals. These data suggest that DNMT3b is not essential for the establishment of maternal imprints, while the association of *DNMT3B* mutations with a human disease with severe chromosome instability (see below) suggests that DNMT3b is important for maintaining chromosome stability by ensuring methylation of repeat sequences.

Studies in humans and other animals

The human *DNMT3* genes are highly homologous to the mouse *Dnmt3* genes[66]. In addition, the same genomic organization and use of transcript variants have also been observed for the human *DNMT3* genes[63,66,69]. *DNMT3A* appears to be ubiquitously expressed, while *DNMT3B* is detected at lower levels in the tissues examined, including testis and ovary[66]. In human oocytes, *DNMT3A* and at least two splice variants of *DNMT3B* are developmentally regulated[58]. Similarly, *DNMT3A* and *DNMT3B* have also been detected in fetal and adult ovaries in the bovine model[60].

Mutations in the human *DNMT3B* gene are associated with human disease, and to date this is the only DNMT known to be causative of human disease when mutated. Various mutations of the *DNMT3B* gene are associated with the genetic disorder known as immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome[67,70]. Among patients with ICF syndrome, cytogenetic abnormalities at centromeric regions of chromosomes 1, 9, and 16 occur due to a loss of methylation on the satellite DNA in these areas[71]. Interestingly, none of the patients are homozygous for null mutations of *DNMT3B*, suggesting that loss of DNMT3B function may be lethal in humans, as it is in mice[62]. These results also support the hypothesis that DNMT3B specifically methylates certain repeat sequences including satellite repeats.

DNMT3L

Mouse studies

Dnmt3L was identified based on sequence similarity to the *Dnmt3* family[49]. Characterization of the genomic organization of the gene revealed that it lacks the catalytic domain, thus it does not likely act as a cytosine methyltransferase[49]. An

in-vitro assay confirmed that DNMT3L appears to lack the ability to methylate DNA[72]. Levels of *Dnmt3L* are low in the female embryonic gonad, and expression peaks in the postnatal ovary[55,64]. High levels of *Dnmt3L* coincide with oocyte growth which suggests a role in maternal imprint establishment[55]. In support of this hypothesis, when *Dnmt3L* was knocked out in mice, oogenesis appeared normal but the deficiency acted as a maternal effect lethal with embryos of homozygous null female mice dying by E9.5 with abnormalities in extraembryonic tissues[50,72]. Analysis of genome wide methylation revealed that global methylation levels appeared normal, but bisulfite sequencing revealed a loss of maternal, but not paternal methylation imprints[50,72]. Since DNMT3L lacks methyltransferase activity, it cannot be solely responsible for establishing the methylation imprints. Co-immunoprecipitation and co-immunolocalization experiments demonstrate that DNMT3L can form a complex with DNMT3a and DNMT3b in vivo[72], suggesting that DNMT3L may be involved in targeting active methyltransferases to imprinted genes in order to establish the methylation imprints in the oocyte.

Studies in humans and other animals

DNMT3L expression has been detected in human testis, ovary, and thymus[49], as well as in preimplantation embryos; however, DNMT3L has not been detected in isolated human oocytes[58]. These differences suggest that there may be differences in maternal imprint establishment between mouse and human oocytes; however, further studies are required.

EPIGENETICS IN ASSISTED REPRODUCTION: QUESTIONS AND CONCERNS

As the number of children born as a result of assisted reproduction increases, there are several aspects of these technologies which are a cause for concern with respect to epigenetic abnormalities. Among these, the association of epigenetic abnormalities with infertility, the impact of aging on epigenetics in the oocyte, the administration of exogenous gonadotropins, and the exposure of both immature oocytes and preimplantation embryos to in-vitro culture are beginning to be acknowledged as possible inducers of epigenetic dysregulation.

Aging oocytes as a cause of infertility

Fertility rates decline rapidly as women enter the fourth decade of life. This decrease in fertility has often been attributed to the loss of the ovarian reserve throughout the reproductive period. The effect of aging on oocyte quality has also been suggested as a reason for the decreasing fertility rates in older women. A single study has examined gene expression in aging oocytes of mice[73]. This study employed carefully designed microarray experiments to compare pooled oocytes from young mice (5–6 weeks) and from mice nearing the end of their reproductive span (42–45 weeks). Of note, this study found significant decreases in the levels of *Dnmt1o, Dnmt1s*, and *Dnmt3L*, and a significant increase in the expression of *Dnmt3b* in older oocytes collected from mice nearing the end of their reproductive lives. This result, while not providing direct evidence of epigenetic dysregulation, suggests that studies are required to examine the effect of aging on the epigenetics of oocytes.

Ovulation induction with gonadotropins

Gonadotropins are often used to stimulate the development and ovulation of multiple oocytes for assisted reproduction. The concern has been raised that this procedure may force oocytes to go through the final growth and maturation process too rapidly or rescue oocytes that might normally undergo atresia,

and as a result oocytes may be of lower quality, or methylation imprints may not be properly established in all oocytes. Additionally, the administration of exogenous gonadotropins may affect the uterine environment due to disruption of the normal levels of endogenous hormones. A single study has examined the effect of superovulation on methylation status[74], however this group only examined the distribution of 5-methylcytosine by immunofluorescence, and not methylation status of specific genetic sequences. Comparison of two-cell embryos from superovulated or natural mating revealed an increase in the number of embryos with abnormal 5-methylcytosine staining patterns among the superovulated group[74].

To address the question of gonadotropin stimulation in humans, prospective or retrospective studies are required with experimental groups who have only undergone ovulation induction, as opposed to the more involved protocols of assisted reproduction. One such study has been reported, in which a large case-control prospective study was undertaken to compare obstetric outcomes of singleton pregnancies in women who had undergone ovulation induction to comparable unstimulated control females[75]. This study described an increased relative risk of gestational diabetes mellitus and pregnancy-induced hypertension. Neither of these illnesses can be attributed specifically to the hormonal stimulation, as the cause may be related to the underlying fertility or treatments[75]. Imprinted genes have yet to be examined.

Further studies are required to examine the effect of gonadotropin stimulation on methylation and expression of imprinted genes. These studies will require careful planning, as there is a decreased rate of implantation and embryonic development in the mouse model[76]. If similar studies are to be undertaken in humans, careful consideration of tissue collection from children conceived using ARTs would be required if the methylation and expression status of imprinted genes are to be examined.

Culture of preimplantation embryos

The effect of preimplantion culture on the methylation and expression of a few imprinted genes has been examined in the mouse[77–79]. Culture in Whitten's media resulted in biallelic expression of *H19*, with a concomitant loss of methylation at a single CpG site upstream of *H19*; however, *Snrpn* was unaffected[77]. Khosla et al.[78] examined relative expression levels of imprinted genes after preimplantation culture in media with or without serum. This group described a decrease in relative expression of *H19, Igf2*, and *Grb7*, and an increase in relative expression of *Grb10* in the serum treated group, however, these changes were not correlated to methylation changes[78]. Another recent study found that preimplantation culture resulted in a decrease in the expression of *H19, Igf2, Peg1*, and *Grb10*[79]. Differences in the results of these studies are likely due to the use of different culture media. Further studies are required to examine the effect of preimplantation culture on the methylation and expression of imprinted genes. These studies are complicated by the requirement to pool preimplantation embryos for expression or methylation studies; however, these problems could be partially averted by transferring cultured embryos to pseudopregnant female mice and allowing the embryos to develop to a stage at which the experiments would be possible on single embryos.

In-vitro growth and maturation of oocytes

In-vitro maturation of oocytes has certain advantages over standard protocols, such as the ability to avoid stimulation by gonadotropins prior to harvesting oocytes, as well as being both less expensive and involving a simpler treatment protocol[80]. The ability to grow and mature oocytes in vitro has important implications for individuals who lose ovarian function early in life, such as cancer patients. There are, however, concerns related to the epigenetics of the oocytes. The first

studies using mouse models of in-vitro growth and maturation have shown that oocytes grown and matured in vitro can generate live offspring, albeit with a very low rate of success[81]. The in-vitro growth and maturation protocol in mouse models has been revised, including changes to media and supplements, and the introduction of a two-step protocol for the culture[82]. The revised protocol resulted in higher levels of live births, although the proportion of live births was still significantly lower than in the control group, derived from in-vivo grown and matured oocytes[82]. Importantly, mouse oocytes grown in vitro do not reach the same diameter as those grown *in vivo*[83,84]. This observation is important, as imprint establishment in mouse oocytes is related to the diameter of the oocyte as opposed to being related to time[32]. In support of this, a recent study described inappropriate methylation of several imprinted genes in oocytes grown and matured in an in-vitro follicular culture model[85]. Although this study was small, together with the other data it suggests that further studies into the effects of in-vitro culture on maternal imprint establishment are required. In pig oocytes, another possible example of epigenetic dysregulation has been described. Oocytes matured in vitro show a decreased rate of proper methylation reprogramming following fertilization in this model[86].

One of the primary advantages of the in-vitro maturation protocol is the avoidance of exogenous gonadotropin treatment[80], however pretreatment, or priming, with either follicle-stimulating hormone (FSH) or human chorionic gonadotropin (hCG) is being suggested by several groups (reviewed in Chian et al.[80]). The administration of exogenous gonadotropins raises the same concerns discussed previously. Additionally, the in-vitro maturation of oocytes requires these cells to be in a culture environment for longer periods of time versus that for IVF or ICSI alone. The extended culture times heighten the concern that epigenetic dysregulation may occur in the synthetic environment. These additional concerns further support the need for detailed studies of the impact of ARTs on the epigenetics of the oocyte.

Assisted reproductive technologies or infertility: what is the cause of epigenetic dysregulation?

Further complicating the question of epigenetic dysregulation as a result of ARTs are two recent reports examining imprinting in subfertile individuals or couples. One group has examined methylation at two imprinted loci in sperm samples from normal fertile males and in oligozoospermic males with moderately or severely reduced sperm counts[87]. When the methylation of *H19*, which is normally hypermethylated in sperm, was examined by bisulfite sequencing, it was observed that both moderately and severely oligozoospermic sperm samples had lower levels of methylation than the normozoospermic controls. Of note, methylation was not completely lost at all sites examined, and the more severely oligozoospermic males exhibited more variability as well as an increased number of sites at which methylation was lost than their moderately affected counterparts. Examination of a maternally methylated gene, *MEST*, showed normal hypomethylation at all sites for all samples, indicating that maternal imprints were correctly erased in these samples[87]. The authors suggest that transmission of imprinting errors may be increased as a result of infertility treatment, although the imprinting errors may not strictly occur as a result of the treatment but may be associated with some forms of male factor infertility.

Further support for a role for epigenetic defects in infertility comes from a retrospective study involving children born with Angelman syndrome in Germany[88]. Parents were contacted and asked to complete a survey related to method of conception and time to pregnancy, as well as to submit tissue samples from parents and child in order to determine the genetic cause of the

Angelman syndrome. As a result, it was discovered that there was an increased incidence of imprinting defects in patients with Angelman syndrome born to subfertile couples, defined as couples with a time to pregnancy longer than two years. The authors further suggest that the imprinting disorder and subfertility may have a common cause, unrelated to ARTs.

These findings support a need for further studies into the effects of ARTs as well as the causes of and epigenetic states in subfertile males and females.

CONCLUSIONS

Important features of the dynamic epigenetic changes that occur during oocyte growth and maturation have been uncovered in recent years. The time of acquisition of maternal methylation imprints may be a stage that is vulnerable to the effects of the different types of ARTs. A growing amount of evidence suggesting an association of imprinting disorders with assisted reproduction highlights the need for further study of epigenetic defects associated with infertility as well as the different aspects of the techniques currently employed. Additionally, testing in animal models should precede the introduction of new treatments and technologies into the clinic setting. The reports linking human imprinting disorders and ARTs suggest that children conceived using these techniques should be followed closely after birth, and highlight the need for large, multi-center prospective studies to examine the incidence of imprinting disorders in this population.

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