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Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development

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Summary

This paper shows stage- and tissue-specific global demethylation and remethylation occurring during embryonic development. The egg genome is strikingly undermethylated and the sperm genome relatively methylated. Following a loss of genomic methylation during preimplantation development, embryonic and extraembryonic lineages are progressively and independently methylated to different final extents. Methylation continues postgastrulation and hence could be a mechanism initiating, or confirming, differential programming in the definitive germ layers. It is proposed that much of the methylation observed in somatic tissues acts to stabilize and reinforce prior events that regulate the activity of specific genes, chromosome domains or the X chromosome (in females). Fetal germ cell DNA is markedly undermethylated and we favour the idea that the germ lineage is set aside before the occurrence of extensive methylation of DNA in fetal precursor cells.

Key words: mouse embryo, DNA, methylation.

Introduction

Methylation of the pyrimidine base cytosine in DNA may be one of the mechanisms underlying differential programming of cell lineages in mammalian development. In somatic tissues approximately 2–5 % cytosines are methylated and the vast majority of these occur in the dinucleotide CpG and hence symmetrically on both DNA strands. Hemimethylated DNA produced by DNA replication becomes fully methylated by the action of a maintenance methylase. Differential patterns of methylation of CpG sites in the genome therefore provide stable and heritable information capable of regulating expression of specific genes (reviewed in Doerfler, 1983), of chromosomes domains (Naveh-Many & Cedar, 1981) and of whole chromosomes (e.g. the active and inactive X chromosomes in female cells, reviewed in Monk, 1986).

Mechanisms must exist that establish different patterns of methylation during embryonic development. Generally the idea of a methylated 'ground state' for development has been favoured (Singer, Robert-Ems, Luthardt & Riggs, 1979; Razin & Riggs, 1980; Jähner & Jaenisch, 1984). This would be in keeping with demethylation being associated with the onset of expression of specific genes. In addition, direct measurement has suggested high levels of methylation in preimplantation mouse (Singer *et al.* 1979) and rabbit (Manes & Menzel, 1981) embryos, as well as in teratocarcinoma cells (Singer *et al.* 1979). Furthermore, Jähner, Stuhlmann, Stewart, Harbers, Löhler, Simon & Jaenisch (1982) have argued for the presence of a *de novo* methylase activity during preimplantation development and in embryonal carcinoma cells (Stewart, Stuhlmann, Jähner & Jaenisch, 1982), to explain the methylation of Moloney murine leukaemia virus sequences following integration into the host genome.

Differential methylation has also been proposed as a basis for the onset of X-chromosome inactivation in female embryos. During development, inactivation of an X chromosome occurs first in extraembryonic lineages as they differentiate and later in embryonic tissue (Monk & Harper, 1979; Takagi, Sugawara & Sasaki, 1982). The preferential inactivation of the

paternal X chromosome in the early extraembryonic lineages (Takagi & Sasaki, 1975; West, Frels, Chapman & Papaioannou, 1977; Harper, Fosten & Monk, 1982) suggests that paternal and maternal genomes are distinguishable (imprinting). During ontogeny of the female germ line the inactive X chromosome is reactivated (Monk & McLaren, 1981). Could differential methylation and changing patterns of methylation be the molecular bases for these events?

In this work we have developed a highly sensitive technique, using only several hundred cells, for looking at genomic DNA methylation. We have examined methylation in oocytes and sperm, preimplantation mouse embryos, different lineages dissected from postimplantation embryos, and purified fetal germ cells, and we have established the timing and pattern of changes in methylation during mouse embryo development.

Materials and methods

Mouse embryo tissues and DNA preparation

All samples were collected in TNE (100 mM-NaCl, 50 mM-Tris pH 7·5, 5 mM-EDTA) containing 2 % sodium dodecyl sulphate and 200 $\mu g\,ml^{-1}$ protease K and stored at −70°C. When sufficient tissue was collected, samples were pooled for DNA preparation.

Sperm was collected from the testis and epididymis. Eggs were collected from female MF1 mice (OLAC) superovulated by intraperitoneal injection of 5 i.u. of PMS (pregnant mare serum) followed 45 to 48 h later by 5 i.u. of HCG (human chorionic gonadotrophin). The following day approximately 1500 ovulated eggs were collected from the oviducts of unmated females. The eggs were treated with hyaluronidase (300 i.u. ml^{-1} in PB1 medium; Whittingham & Wales, 1969) for 5 min to remove cumulus cells. Residual adhering cumulus cells were removed by finely drawn pipettes.

From mated females approximately 500 8-cell embryos were flushed from the oviducts on the third day of pregnancy, and around 200 blastocysts from the uterine horns on the fourth day of pregnancy. Approximately 60 postimplantation embryos at 6½ and 7½ days' gestation were dissected from the uteri of naturally mated females. The egg cylinders were treated with trypsin and pancreatin to loosen the cells layers. The 6½-day egg cylinders were dissected into epiblast, extraembryonic ectoderm and primary endoderm as described in Monk & Harper (1979), and the 7½-day egg cylinders into embryonic and extraembryonic (chorion) portions.

From 12½-days' gestation gonads can easily be recognized as male or female; male gonads show developing testicular cords. At 11½ days, embryos must first be sexed by examination of amnion cell nuclei for sex chromatin (Burgoyne, Tam & Evans, 1983) to identify gonads as male or female (part of this work was kindly performed by Mr Phillip Lee). At least 30 gonads, together with attached mesonephros tissue, were isolated from postimplantation embryos from 11½ to 16½ days' gestation and the gonad and mesonephros were dissected apart. Embryonic kidneys, extraembryonic placentae and yolk sacs were also isolated. The mesoderm and endoderm components of the yolk sac were separated as described in McMahon, Fosten & Monk (1983).

Approximately 90 % pure populations of germ cells were obtained from isolated 12½- and 14½-day female and male gonads as follows. Gonads were incubated in phosphate-buffered saline, containing 0·4 % polyvinylpyrrolidone and 0·2 % EDTA, at room temperature for 30 min. The germ cells were released by rupture and squeezing of the gonads and purified by gently wafting them away, in a stream of medium, from gonadal somatic cells and red blood cells, keeping them always grouped together. They were collected into microcaps for storage at −70°C. Germ cells were collected in this way from between 150 and 200 gonads for each sample. We are grateful to Susan Lindsay for help with the collections.

For the larger amounts of tissue, DNA was isolated as described in Flavell, Kooter, de Boer, Little & Williamson (1978). DNA was prepared from sperm nuclei according to Bird, Taggart & Gehring (1981). For the samples of low cell numbers, DNA was isolated by ultracentrifugation as follows. Samples stored at −70°C were pooled, the volume made up to 2 ml with TNE (2 % SDS, 200 $\mu g\,ml^{-1}$ protease K, see above), incubated at 37°C for 1 h (to allow protease K digestion), phenol-extracted two times and two volumes of absolute alcohol added to the aqueous phase. Following ultracentrifugation (25 000 rev min^{-1}, 18 h) the deposit was gently washed with 70 % alcohol, resuspended in 30 μl of Tris 10 mM pH 7·4, EDTA 1 mM, RNase 25 $\mu g\,ml^{-1}$, and stored at −20°C ready for use.

To ensure the reproducibility of results, collections of biological material and DNA preparations were made on at least two separate occasions for each stage of embryonic development, except the pure germ cell isolations that were performed once only.

End-labelling

DNA preparations were digested for 1 h with *Msp*I or *Hpa*II (enzymes from BioLabs). Completeness of digestion was assured by the use of excess of enzyme (about 100-fold) on the minute quantities of DNA involved. Completeness of digestion and lack of degradation was supported by the reproducibility of the results from replicate experiments with independent DNA preparations, and the appearance of bands representing repetitive sequences or mitochondrial DNA (see Results). The digests were end-labelled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (Maniatis, Fritsch & Sambrook, 1982). Uncut (mock-digested) DNA was end-labelled in each experiment to ensure the high molecular weight of the starting DNA preparations and verify the absence of degradation (e.g. see Fig. 1). A *Hin*dIII digest of 5 ng of λ DNA was end-labelled to serve as markers. The end-labelled DNA was subjected to electrophoresis (16 h, 25 V) on a 0·7 % agarose gel (0·5 to 1 ng DNA per slot) and Southern blot (Southern, 1975) of the labelled fragments. The fragment size distribution could be observed following exposure of the filters to X-ray film (about 3 days). Transfer of DNA from the 0·7 % gels to the

filters was complete since only background counts were detectable in the gel following blotting.

Statistical evaluation

Densitometry was performed with a Joyce Loebl Chromoscan 3. Variation of degree of methylation is correlated with both the total number of fragments (labelled ends) as well as the shape of the distribution of fragments at the tops of the lanes. Comparisons of total counts as an indication of number of fragments proved unreliable due to uneven loading of the minute quantities of DNA in some of the gels and the differences being confined mainly to the tops of the lanes. However, a measure of the shapes of the *Hpa*II fragment distributions of the different DNAs could be obtained from the ratio of number of fragments at positions 10 and 3 kb. The 10/3 ratios for the different DNAs were normalized to the 10/3 ratio of a reference DNA on each gel. Results determined in this way were reproducible for replicate experiments for independent isolates of a particular tissue DNA.

Hybridization

DNA preparations were digested with *Msp*I and *Hpa*II. Completion of digestion was monitored by digestion of λ phage DNA added to a sample of the main digest. Digests were loaded at 0·1 μg per lane on 0·7 % agarose gels and electrophoresed for 16 h at 25 V. Following transfer of the DNA fragments to nitrocellulose by Southern blot, they were hybridized to ^{32}P-labelled nick-translated probe (pMR134 or mitochondrial probe, 200 ng, approximately 2×10^8 dissints min^{-1} μg^{-1}) for 48 h, the filters were washed and exposed to X-ray film for approximately 1 week.

Results

Differences in global DNA methylation in gametes and early embryos

DNA was isolated from mature sperm, ovulated unfertilized oocytes, 8-cell embryos, blastocysts and separated embryonic and extraembryonic lineages of postimplantation conceptuses as described in the Materials and methods. To study methylation, DNA preparations were digested with the isoschizomeric enzymes *Msp*I and *Hpa*II which cut the DNA at the four-base sequence, CCGG. *Msp*I will cleave the DNA at this sequence whether the internal cytosine is methylated or not, whereas *Hpa*II will only cut the unmethylated sequence (Waalwijk & Flavell, 1978*a*). A comparison of *Msp*I and *Hsp*II fragment sizes therefore gives an indication of the extent of methylation of the total genomic DNA. Fragment-size distributions could be observed by end-labelling the DNA fragments with [α-^{32}P]dCTP, using the Klenow fragment of DNA polymerase I, followed by electrophoresis, blotting to nitrocellulose filters and autoradiography. The method is extremely sensitive and global methylation can be examined in 1 ng of DNA (from approximately 200 cells). It must be borne in mind in interpreting these experiments that the end-labelling procedure is an indication of numbers of fragments of a particular size and not the molar amount of DNA in the different regions of the gel. The high M_r DNA is therefore under-represented since ends are so few. Nevertheless the results are reproducible and the technique allows comparisons between different DNA isolates.

Fig. 1 shows a representative autoradiograph of end-labelled uncut samples of the DNA preparations used in this work. The low level of labelling at the top of the lanes indicates large fragments and few ends and that therefore the starting DNA is high M_r. Very slight degradation (labelled ends of low M_r fragments at the bottom of the lanes) is seen for some of the DNAs.

Fig. 2 shows a representative experiment with end-labelled embryonic DNAs following *Msp*I and *Hpa*II digestion. The heavy labelling at the bottom of all the

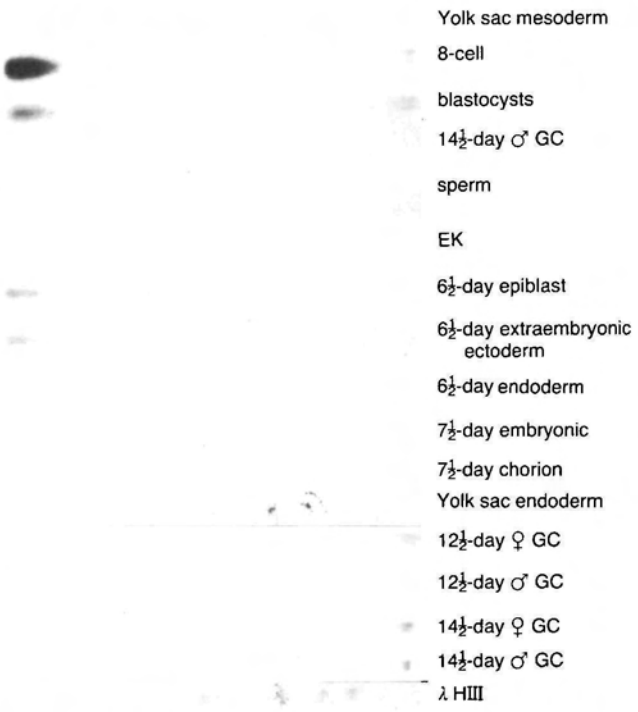

Fig. 1. End-labelling of the starting DNA preparations from yolk sac mesoderm, 8-cell embryos, blastocysts, sperm, embryo-derived stem cells (EK), 6½-day epiblast, 6½-day extraembryonic ectoderm, 6½-day endoderm, 7½-day embryonic region, 7½-day chorionic region, yolk sac endoderm, 12½-day ♀ germ cells (GC), 12½-day ♂ GC, 14½-day ♀ GC and 14½-day ♂ GC. In each experiment DNAs were mock-digested (addition of 1 μl of buffer instead of enzyme) for 1 h and end-labelled. Following electrophoresis and blotting, filters were exposed to X-ray film for times equivalent to those used for the digested samples. The low level of labelling at the tops of the lanes in this representative gel indicates that the DNAs were high relative molecular mass at the outset and were not degraded by the experimental procedures used.

lanes is due to the high proportion of fragments derived from the islands of clustered CpG sequences known to occur upstream from many genes (Bird, Taggart & Gehring, 1985). These islands are unmethylated (except on the inactive X chromosome). An indication of the degree of methylation of the total DNA of a particular tissue may be observed by a comparison of *Msp*I and *Hpa*II digests at the tops of the lanes; methylated DNA is indicated by a distribution of the label skewed towards the higher M_r in the *Hpa*II lanes. Densitometry tracings were made from all replicate gels and a measure for comparison of fragment-size distribution in the different DNAs was obtained from the ratio of fragments at positions 10 and 3 kb (indicated in Fig. 2). According to this criterion (see below and Table 1) the sperm and 8-cell DNA are more methylated than oocyte DNA, and the blastocyst DNA shows very low methylation indeed.

For the separated lineages of the postimplantation conceptuses, also shown in Fig. 2, the DNA of the 6½-day embryonic region (epiblast) shows only a slight skewing of label towards high M_r methylated fragments. One day later, the DNA of the embryonic region of the 7½-day conceptus is highly methylated. The DNAs of the extraembryonic lineages in the postimplantation conceptuses, the primary endoderm (and extraembryonic ectoderm, data not shown in Fig. 2) at 6½-days' gestation, and the chorion at 7½-days' gestation, are more methylated than blastocyst DNA, yet undermethylated compared with the 7½-day embryonic tissue DNA (and see Table 1).

The repetitive DNA fragments seen in the oocyte DNA, and to a lesser extent in the 8-cell and blastocyst DNAs in Fig. 2, are *Msp*I fragments from mitochondrial DNA. Fig. 3 shows an *Msp*I digest of liver DNA hybridized with a mixture of three probes together covering the entire mitochondrial DNA (Kearsey, Flannagan & Craig, 1980). The band pattern agrees well with that observed for the repeated fragments in the end-labelled total DNA of the oocytes and preimplantation embryos.

Global DNA undermethylation in fetal germ cells

During mouse fetal development the primordial germ cells reach the developing gonads at around 11 days' gestation and by 13 days' gestation female germ cells have entered into the prophase of the first meiotic division. The previously inactive X chromosome is reactivated by about 13 days' gestation (Monk & McLaren, 1981). At this time male germ cells have

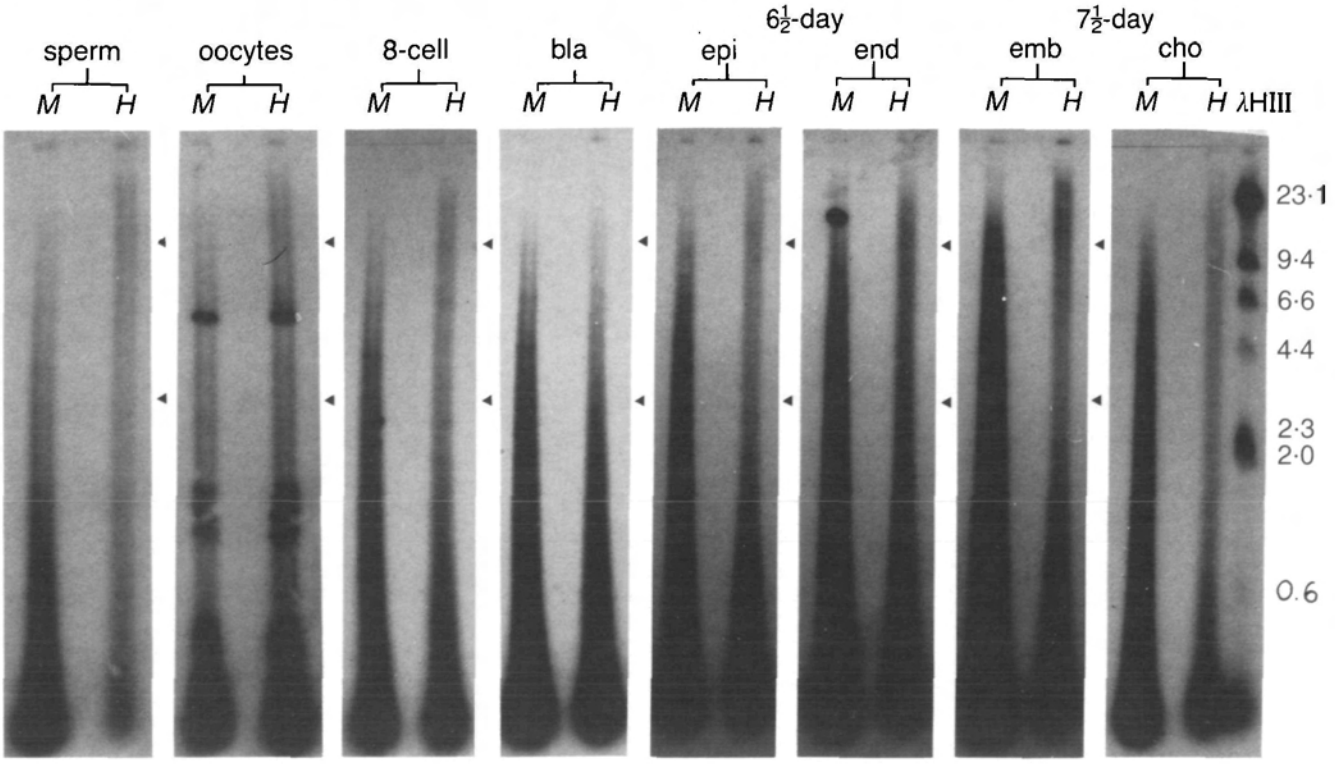

Fig. 2. Changes in methylation in development. DNAs from sperm, oocytes, 8-cell embryos, blastocysts (bla) and dissected lineages of postimplantation conceptuses (6½ days: epi, epiblast, end, primary endoderm; 7½ days: emb, embryo, cho, chorion) were digested with *Msp*I(*M*) and *Hpa*II(*H*) and end-labelled as described in Materials and methods. The figure shows a representative experiment from eight independent replicate experiments. The repetitive bands in the oocyte DNA are mitochondrial in origin (see Fig. 3).

Table 1. *DNA methylation in development – log ratios of fragments at 10 kb and 3 kb in* Hpa *II digests of DNA of tissues, arranged in order of decreasing methylation*

Tissue DNA	Log mean* ± S.E.M.
7½-day embryo	0·307 ± 0·089
yolk sac mesoderm	0·274 ± 0·093
EK cell	0·172 ± 0·075
sperm	0·067 ± 0·037
8-cell embryo	0·039 ± 0·061
6½-day epiblast	0·035 ± 0·087
7½-day chorion	0·035 ± 0·080
6½-day extraembryonic	−0·089 ± 0·050
oocytes	−0·210 ± 0·049
14½-day male germ cells	−0·286 ± 0·045
12½-day male germ cells	−0·287 ± 0·036
12½-day female germ cells	−0·310 ± 0·040
blastocyst	−0·349 ± 0·046
14½-day female germ cells	−0·483 ± 0·018

* Values of 10 to 3 kb ratios for each gel are normalized to $\log_{10}$ values of 6½-day primary endoderm or yolk sac endoderm on the same gel taken as zero.

entered mitotic arrest; further rounds of mitosis and subsequent entry into meiosis will begin after birth.

In order to examine the methylation status of the fetal germ cell DNA during the period of X-reactivation and initiation of meiosis in the female germ cells, DNA was prepared from female and male fetal germ cells at 12½ and 14½ days' gestation and from whole gonads, containing both germ cells and supporting somatic cells from 11½ to 16½ days' gestation. In addition, DNA was prepared from 16½-day embryonic somatic tissue, the mesonephros tissue (dissected apart from the developing gonad) and kidneys, from extraembryonic tissues (yolk sac endoderm, yolk sac mesoderm and placenta), and from an embryo-derived stem cell line (HD14, Evans & Kaufman, 1981).

Fragment size distributions are shown in Fig. 4 for *Msp*I and *Hpa*II digests of the DNA from 12½- and 14½-day female and male germ cells, from DNAs of the separated yolk sac endoderm and mesoderm components and from embryo-derived stem cells. Once again the majority of ends are generated by the *Hpa*II tiny fragments from the methylation-free islands of CpG sites in both the *Msp*I and the *Hpa*II digests. Densitometry traces were taken from all replicate gels and the traces measured at positions 10 and 3 kb. This analysis (see below, and Table 1) shows that the fetal germ cell DNAs are markedly undermethylated compared to the yolk sac DNAs, and all other DNAs.

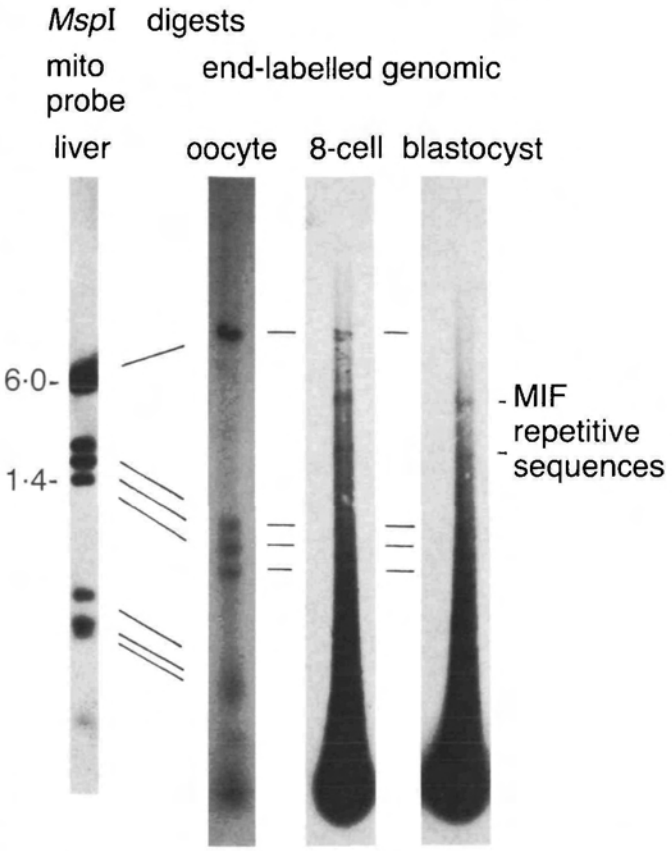

Fig. 3. Mitochondrial DNA in preimplantation embryos. End-labelled, *Msp*I-digested DNAs from oocyte, 8-cell embryos and blastocysts showing that the repetitive bands are equivalent to those identified by hybridization of an *Msp*I digest of adult liver DNA to the mouse mitochondrial probe. Repetitive MIF DNA sequences are also indicated.

Statistical evalulation of global methylation

Quantification of absolute levels of methylation in the different tissues is not possible due to the bulk of the fragments from the CpG islands. Nevertheless comparisons between tissues could be made by analysis of ratios of fragments at positions 10 and 3 kb in the *Hpa*II lanes. After logarithmic transformation to reduce the dependence of variance on the mean, the data were analysed by a sequential modification of the Q method for analysis of variance (Snedecor & Cochran, 1967). Table 1 summarizes the data obtained from replicate analyses of the DNAs with the log mean values arranged in descending order of degree of methylation. The data are reproducible and show different levels of DNA methylation with respect to skewing of the *Hpa*II distributions towards heavier fragments. The most methylated DNA is 7½-day embryonic DNA. DNAs from 6½-day epiblast, from extraembryonic portions of 6½- and 7½-day conceptuses and from 8-cell embryos are significantly less methylated than 7½-day embryonic DNA. DNAs

from oocytes, blastocysts and fetal germ cells are strikingly undermethylated and significantly less methylated than all the other DNAs, including sperm DNA.

There were no significant differences between tissues for the ratios of fragments at 10 and 3 kb in the *Msp*I digests. In addition to the comparison of *Hpa*II lanes alone, a further analysis was done to compare values for the *Hpa*II ratio divided by the *Msp*I ratio from the accompanying *Msp*I lane for each DNA in each experiment. Although this analysis showed greater variation in replicate pairs of lanes than did the analysis of the *Hpa*II lanes alone, the *Hpa*II 10/3:*Msp*I 10/3 values showed the same rank order as the *Hpa*II ratios in Table 1.

Methylation of repetitive sequence, MIF, in somatic and germ cell DNA

The fetal germ cells are markedly undermethylated and this could be in part the molecular basis for the X-chromosome reactivation. In this respect, the interesting question is whether there is a specific demethylation event in the germ line. There is evidence from dosage of hypoxanthine phosphoribosyl transferase activity (Monk & McLaren, 1981) and expression of phosphoglycerate kinase isozymes (McMahon, Fosten & Monk, 1981), that the inactive X chromosome may already be reactivated by 12½ days' gestation. To analyse methylation of DNA from pure germ cells earlier, at 11½ days' gestation, would be difficult, not only because each embryo must be individually sexed by sex chromatin in the amnion, but also due to the difficulty in isolating sufficient germ cells. We therefore decided to look at methylation of the DNA in whole gonads in the region of a dispersed repetitive sequence, MIF (mouse interspersed family, recognized by probe pMR134, Bennet, Hill, Pietras, Woodworth-Gutai, Kane-Haas, Houston, Heath & Hastie, 1984, approximately 3×10^4 copies of 5–7 kb per haploid genome). The probe was kindly provided by Nick Hastie (MRC Clinical and Population Cytogenetics Unit, Edinburgh).

Fig. 5 shows hybridization of probe pMR134 to the DNA preparations of the different tissues of the female and male conceptuses and also to sperm DNA, and DNA from an embryo stem cell line. The *Msp*I lanes show two bands of hybridization whereas *Hpa*II digests of DNAs from somatic tissues, female and male embryonic kidneys and mesonephros,

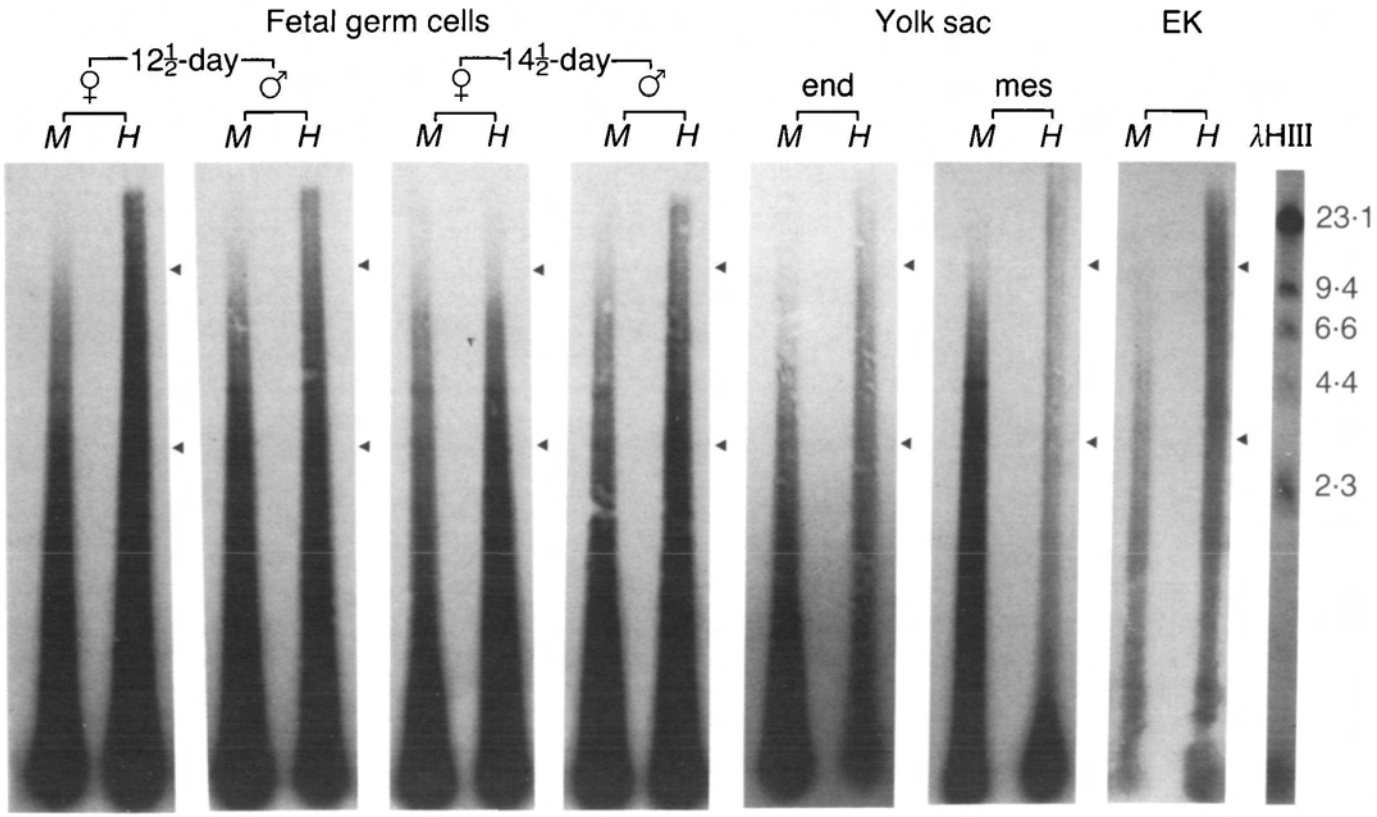

Fig. 4. Undermethylation of fetal germ cell DNA. DNA from purified germ cells, female (♀) and male (♂), at 12½ and 14½ days' gestation, and from yolk sac endoderm (end) and mesoderm (mes) and embryo-derived stem cells (EK), digested with *Msp*I(*M*) and *Hpa*II(*H*) and end-labelled as described in the Materials and methods. The figure shows a representative experiment from eight independent replicate experiments. The 14½-day female germ cell DNA is less methylated but we cannot exclude the possibility that the difference is due to less somatic cell contamination.

show a high molecular weight smear of hybridizing DNA. Therefore the MIF dispersed repeat sequence detected by pMR134 is methylated in somatic tissues. Placental DNA and yolk sac endoderm DNA show a *Hpa*II pattern indicating reduced methylation with a smear extending down to clearly visible bands corresponding to those in the *Msp*I lanes. Yolk sac mesoderm DNA however is methylated to a similar extent to somatic DNA. These results confirm work already published by others (Chapman, Forrester, Sanford, Hastie & Rossant, 1984) and are included here for comparison of methylated and undermethylated DNAs with the gonadal DNA and to show that tissue-specific differences in methylation estimated from end-label analysis are supported by the findings with the repetitive sequence.

Both female and male gonad DNA from 12½-day embryos show the unmethylated bands as well as methylated DNA at the top of the lanes. At 16½ days' gestation the female gonads continue to show an unmethylated component in the *Hpa*II digests. The unmethylated bands appear fainter for the 16½-day male gonads and for mature sperm DNA. Embryo-derived stem cell (EK) DNA shows an undermethylated pattern similar to yolk sac endoderm DNA. Similar tissue-specific differences in methylation were obtained for a satellite repetitive sequence (data not shown; Sanford, Forrester, Chandley & Hastie, 1984).

To show that the unmethylated component of the gonad DNA was due to the germ cells, some of the purified germ cell DNAs were used to hybridize to the MIF probe. Fig. 6 shows that the purified germ cell DNAs show predominantly the unmethylated bands with probe pMR134. There is little hybridization at the top of the lanes in the absence of DNA from the somatic component of the gonads. Having thus established that the unmethylated MIF sequences in the gonad could be ascribed to the germ cells we analysed DNAs from gonads isolated from embryos from 11½ through 16½ days' gestation. The results are shown in Fig. 7. Although the hybridization of the 11½-day DNAs is poor due to paucity of DNA and long exposure to X-ray film it is nevertheless clear that 11½-day germ cells are already unmethylated for these repeat sequences.

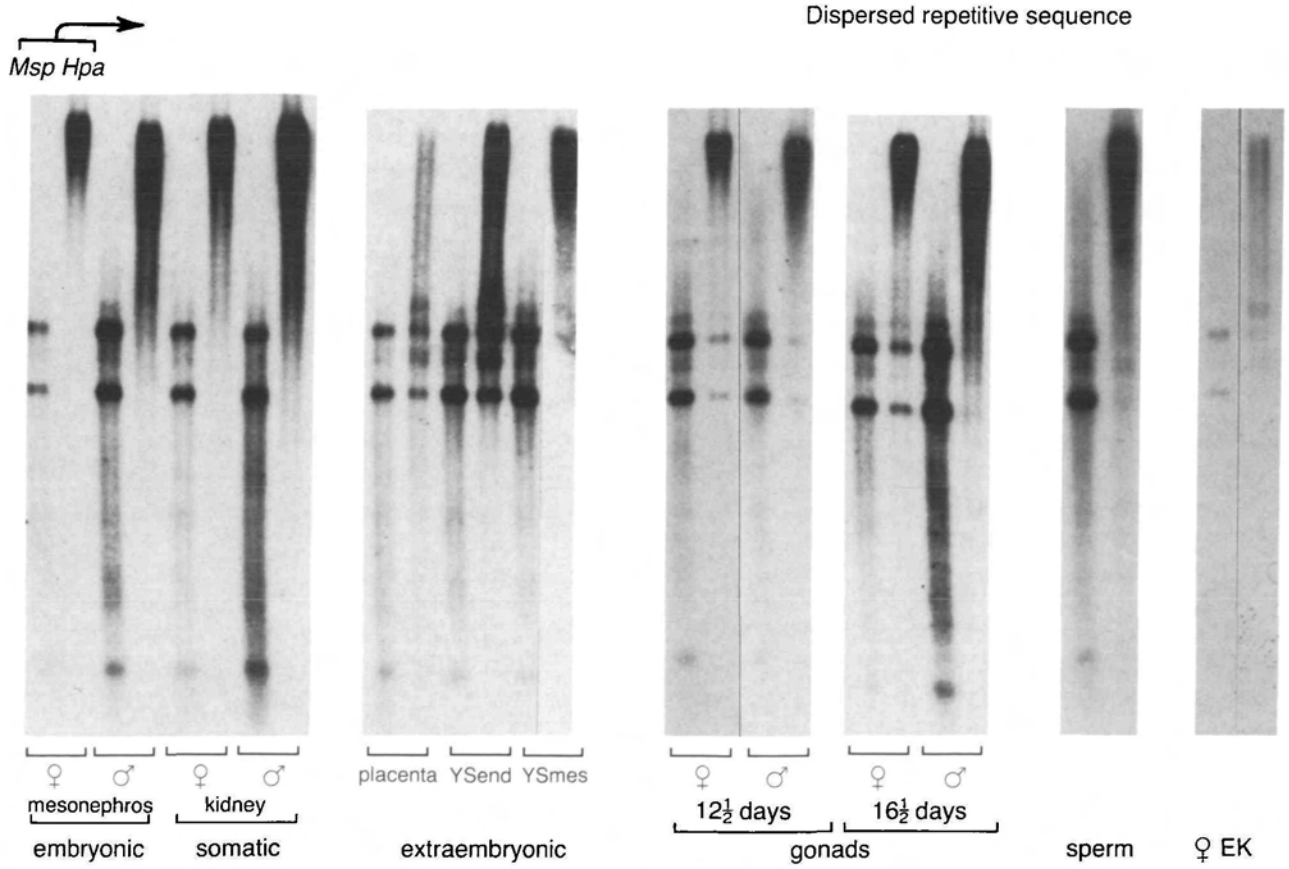

Fig. 5. Methylation of MIF sequences in different embryonic tissues. DNAs isolated from embryonic kidneys, mesonephros, placenta, yolk sac endoderm and mesoderm, female and male gonads at 12½ and 14½ days' gestation, mature sperm and EK cells, were digested with *Msp*I and *Hpa*II, subjected to electrophoresis, Southern blotted to filters and hybridized to the mouse repetitive probe pMR134.

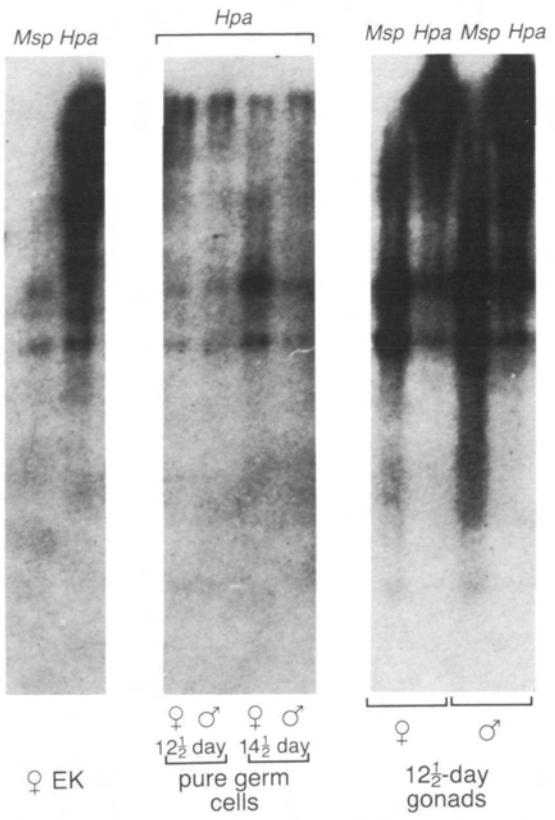

Fig. 6. Undermethylation of MIF repetitive sequences in fetal germ cells. DNA from EK cells, purified germ cells and gonads, digested with *Msp*I and *Hpa*II as shown and hybridized to pMR134. *Hpa*II lanes for purified female (♀) and male (♂) germ cells show only the unmethylated bands indicating that the unmethylated bands seen for gonadal DNAs are due to the germ cell component of the whole gonads.

Discussion

We have developed techniques for the isolation of micro-amounts of DNA from early embryonic lineages and subsequent analysis of overall genomic DNA methylation by end-labelling. The end-label technique allows comparisons of overall genomic DNA methylation in the DNA of as few as 200 cells and could be extended to other areas where biological material is limiting such as stem cell systems and small pieces of tissue in fate-mapping studies. The quantification of the shape of the *Hpa*II fragment distributions by ratios of fragments at 10 and 3 kb is to some extent arbitrary but nevertheless it is reproducible and allows an objective measure of tissue- and stage-specific differences. The validity of the quanitification of methylation is supported by similar tissue-specific differences in DNA methylation obtained by hybridization techniques (this work; Chapman *et al.* 1984; Sanford *et al.* 1984; Sanford, Chapman & Rossant, 1985) and by direct measurement (Razin, Webb, Szyf, Yisraeli, Rosenthal, Naveh-Many, Sciaky-Gallili & Cedar, 1984).

The experiments described in this paper relate to several areas in development: imprinting of maternal and paternal genomes, demethylation and remethylation in early development, independent methylation of embryonic and extraembryonic lineages, X-inactivation and reactivation and the origin of the germ line. These aspects are discussed below.

(A) *Oocyte DNA is undermethylated, sperm DNA is methylated*

Sanford *et al.* (1984) previously demonstrated undermethylation of immature oocyte DNA and methylation of sperm DNA for the dispersed repetitive sequences (MIF) and undermethylation of satellite sequences for both genomes. We have confirmed and extended their results to show global genomic undermethylation of oocytes and (relatively) global genomic methylation of sperm. In addition we have used ovulated oocytes that have completed the first meiotic division and therefore represent more accurately the maternal genome at the time of fertilization. Sperm DNA is known to be highly methylated in the region of a number of unique sequences (Waalwijk & Flavell, 1978*b*; Mandel & Chambon, 1979; Rahe, Erickson & Quinto, 1983). On the other hand sperm DNA appears to be unusual in that satellite sequences are undermethylated (Sturm & Taylor, 1981; Sanford *et al.* 1984; Ponzetto-Zimmerman & Wolgemuth, 1984; Monk, unpublished data). Overall, in our analysis, sperm DNA is undermethylated compared to DNA of somatic tissues (see also Razin *et al.* 1984), similar to DNA of extraembryonic tissue, and methylated compared to DNAs of oocytes, fetal germ cells and blastocysts. The difference in the degree of methylation of the maternal and paternal genomes at the onset of development suggests methylation as a good candidate for differential imprinting.

(B) *The sperm genome is demethylated during cleavage*

DNA methylation in the 8-cell embryo is compatible with a mixture of undermethylated maternal and methylated paternal DNA. There may be some maintenance methylase inherited in the egg cytoplasm but this appears to be lost or diluted out during cleavage since blastocyst DNA has very little methylation. The loss of sperm genome methylation could be one aspect of the activation of the paternal X-linked hypoxanthine phosphoribosyl transferase (HPRT) gene during preimplantation development (Kratzer & Gartler, 1978; Monk & Harper, 1978).

(C) *The extraembryonic trophectoderm and primary endoderm genomes are methylated independently and to a lesser extent than the embryonic genomes*

It has been proposed that a demethylation process is associated with the delineation of extraembryonic tissues (Manes & Menzel, 1981; Razin *et al.* 1984; Sanford *et al.* 1985). In this study we show that trophectoderm, and probably primary endoderm, are delineated at a stage when there is very little of the DNA methylation inherited in the sperm and oocyte genomes remaining. The blastocyst trophectoderm DNA, and also the primary endoderm DNA, must however become further methylated subsequently. Therefore, their patterns of methylation occur *independently* and *potentially differently* from those in the embryonic genomes. Overall genomic methylation in these extraembryonic lineages however does not occur to the final extent observed in the fetal somatic tissues. This has been previously reported in the mouse for total DNA (Razin *et al.* 1984) and for repetitive sequences (Chapman *et al.* 1984; also this paper) as well as for a number of unique gene sequences (Razin *et al.* 1984; Sanford *et al.* 1985) and in human, where global methylation of DNA of the placenta is less than that for somatic tissue DNA (Lindsay, Adams and Monk, unpublished data).

(D) *In fetal precursor cells DNA methylation is detectable in the 6½-day epiblast but is much increased by 7½ days' gestation*

The onset of methylation in the fetal lineage probably begins around the time of implantation and occurs progressively over several cell generations. Recent work has shown that DNA from the ICM of the blastocyst of the mouse on the fourth day of pregnancy is not detectably methylated (Monk, Boubelik & Maidens, in preparation) but that onset of methylation is detectable one day later and increases progressively thereafter (this work). It has been suggested (Monk, 1986) that 'reinforcing' methylation may be superimposed on prior events regulating activity of genes or chromatin domains so as to stabilize or irreversibly inactivate regions that will never be required for a particular differentiated cell lineage. In fetal genomes much of the methylation occurs post-gastrulation and hence methylation could be involved in the differential programming of the definitive germ layers.

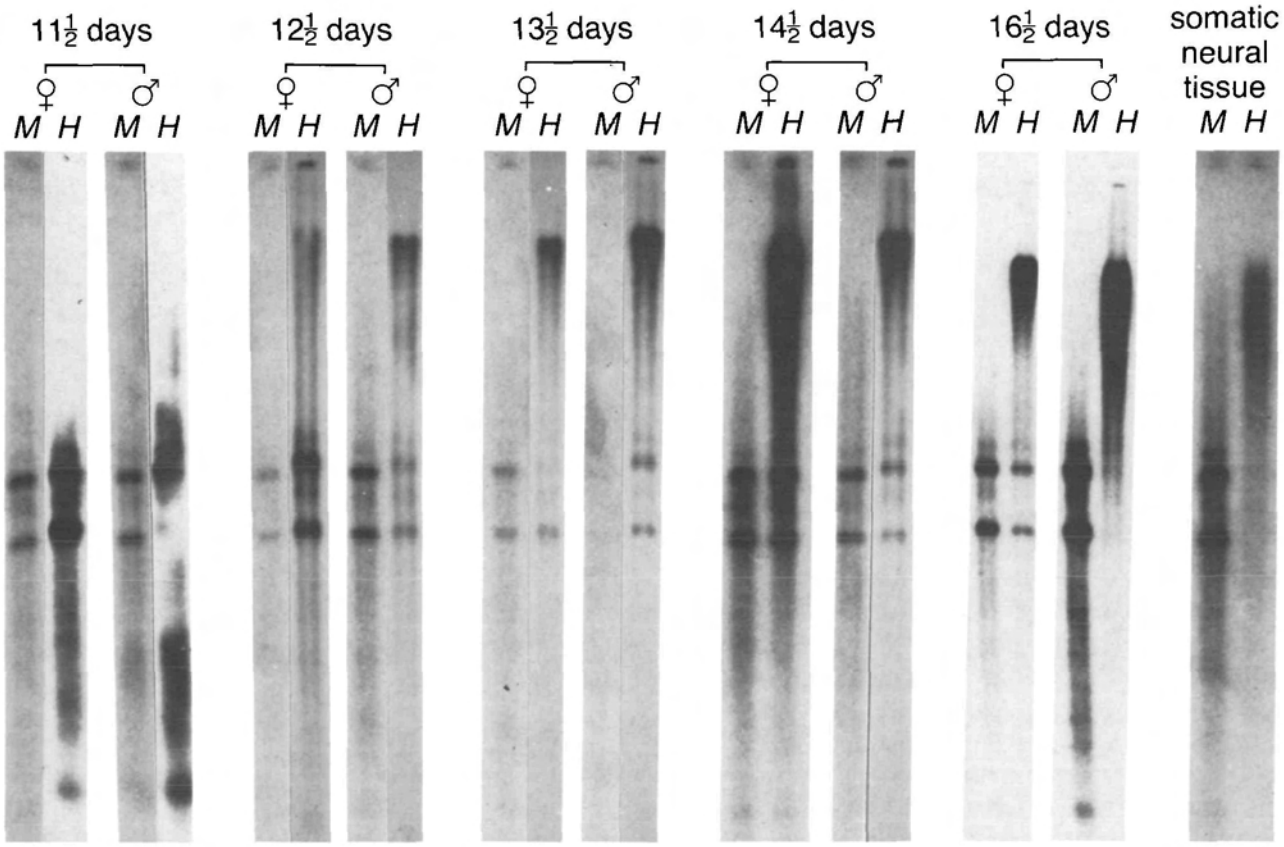

Fig. 7. Undermethylation of MIF repetitive sequences in mitotic and meiotic fetal germ cells. DNAs from embryonic gonads from 11½ to 16½ days' gestation digested with *Msp*I(*M*) and *Hpa*II(*H*) hybridized to repetitive sequence probe pMR134. The unmethylated MIF sequences are seen throughout these stages of development in the germ cells except at 16½ days' gestation in the male gonads and in the control somatic neural tissue DNA.

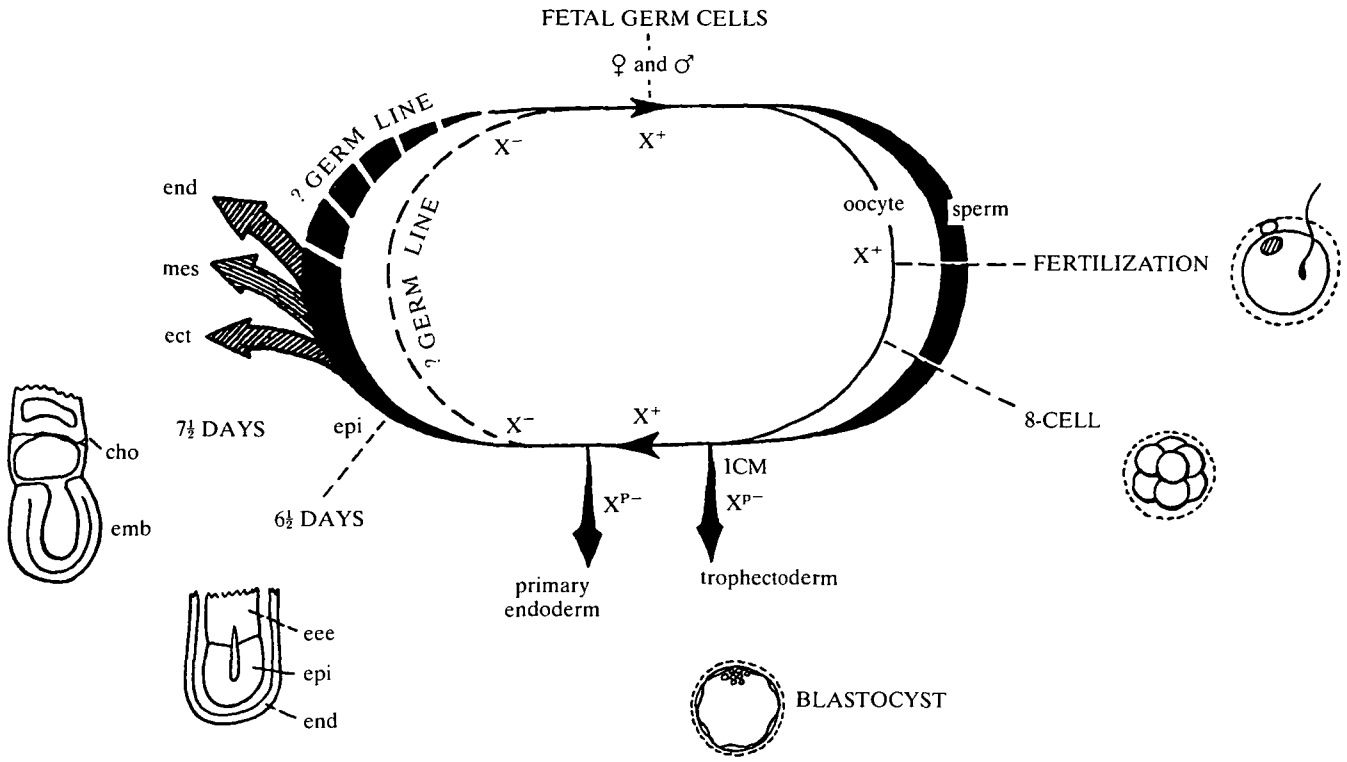

Fig. 8. A model for DNA methylation in development. Increasing (*de novo*) methylation is shown by a line of increasing thickness, decreasing methylation by a line of decreasing thickness, and undermethylation by a single straight line. The definitive germ layers, ectoderm (ect), mesoderm (mes) and endoderm (end), and the extraembryonic tissues, are patterned differently to indicate the possibility of different patterns of methylation. X-inactivation precedes the delineation of the germ layers and the germ line (McMahon *et al.* 1983) and occurs in fetal precursor cells around the time of onset of *de novo* methylation (this paper). Two alternative pathways for the origin of the germ line are shown by dashed lines. The early departure of a markedly undermethylated germ line which escapes further 'reinforcing methylation' (see text) is favoured. ICM, inner cell mass; epi, epiblast; eee, extraembryonic ectoderm; cho, chorionic region.

(E) *X-chromosome inactivation and reactivation*

The early X-inactivation in the extraembryonic tissues does not appear to be linked to a global methylation event. Rather, these tissues arise at a time when there is little methylation remaining. The preferential inactivation of the paternal X chromosome could be related to the higher methylation of the sperm genome compared to that of the oocyte, and residual methylation differences in the paternally inherited X chromosome at the blastocyst stage.

The initiation of X-chromosome inactivation in the embryonic region may coincide with the onset of *de novo* methylation in this tissue but further methylation follows and the primary X-inactivation event may well be initiated by other mechanisms.

The extremely low methylation in fetal germ cells may be one aspect of the molecular basis of X-chromosome reactivation in that the low methylation condition may allow, or enable, reactivation. However, a specific demethylation event as the basis for X-chromosome reactivation does not appear to occur; as early as 11½ days' gestation, prior to X-reactivation, fetal germ cells are already undermethylated.

(F) *The origin of the germ line*

Fig. 8 shows a working model for stage- and tissue-specific demethylation and *de novo* methylation in early development. Strikingly undermethylated DNA is depicted by a single line, and increasing and decreasing methylation as changing thickness of line. The two alternative paths for the origin of the germ line are shown as interrupted lines – one prior to *de novo* methylation, the other involving methylation and demethylation. We favour the hypothesis that the germ line delineates independently prior to the delineation of the three germ layers, and prior to any extensive methylation of the fetal precursor cell genomes. First, at the earliest stage we have examined, germ cell DNA is already undermethylated. Second, on theoretical grounds, the germ line genome must not be subjected to 'reinforcing methylation' since it must remain totipotent, or permissive to reprogramming to developmental totipotency.

(G) *Conclusions*

In early mouse development, following loss of methylation of the paternal genome DNA, methylation occurs independently and to different extents in the primary germ layers, the extraembryonic lineages and in the germ line. The onset of methylation in development will be regulated by the timing of activation of the embryonic genes for maintenance methylase and the extent of methylation may be determined by an interplay of lineage-specific chromatin structure and methylase activities. Extensive methylation may be a mechanism of reinforcing or stabilizing active and inactive chromatin (e.g. the inactive X chromosome) and the germ cell lineage may well be delineated before this occurs.

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