Histone methylation defines epigenetic asymmetry in the mouse zygote

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ABSTRACT The oocyte cytoplasm regulates and enhances the epigenetic asymmetry between parental genomes and, consequently, functional differences observed between them during development in mammals. Here we demonstrate a preferential interaction of HP1β with the maternal genome immediately after fertilisation in the mouse zygote, which also shows a high level of lysine 9-methylated histone H3. In contrast, the paternal genome has neither HP1β binding nor methylated histone H3 at these early stages. Paternal binding of HP1β is only detected at the pronuclear stage, prior to the appearance of lysine 9-methylated histone H3. The early recruitment of heterochromatic factors specifically to the maternal genome could explain the preferential DNA demethylation of the paternal genome in the zygote.

KEY WORDS: *Zygote, imprinting, histone methylation, epigenetic, HP1*

The oocyte cytoplasm has played a major role during the evolution of diverse reproductive strategies. This was probably the case in shaping the epigenetic differences between parental genomes during the evolution of genomic imprinting in mammals, which is responsible for functional differences between parental genomes during development. These functional differences are due to the monoallelic expression of different imprinted genes from the parental genomes (Ferguson-Smith and Surani, 2001). The epigenetic differences between the parental genomes are enhanced in the zygote by means of DNA demethylation of the paternal genome shortly after fertilisation, while the maternal genome displays *de novo* methylation (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Santos *et al.*, 2001). This demethylation of the paternal genome has been observed in all mammalian species studied to date (Dean *et al.*, 2001), but not in animals that do not exhibit genomic imprinting, such as zebrafish (Macleod *et al.*, 1999). Such opposite effects on the parental genomes within the same oocyte cytoplasm might be achieved by the differential binding of stored cytoplasmic factors to the parental genomes. Here we have examined such a role for the heterochromatin protein HP1β which has been shown to interact with histone H3 methylated at lysine 9 (Bannister *et al.*, 2001).

First, we find a high level of lysine 9-methylated histone H3 (meH3) on the arrested maternal chromosomes prior to fertilisation (Fig. 1, B-D). This high level is maintained on the two separating haploid maternal genomes following fertilisation (Fig. 1, H-J), as development commences with the extrusion of the second polar body. Immediately after fertilisation, HP1β protein that is stored in the oocyte cytoplasm binds preferentially and exclusively to the maternal genomes (Fig. 1 K-M). Prior to fertilisation, HP1β was detected in the oocyte cytoplasm by western blotting (data not shown), but none could be detected as being bound to the arrested maternal chromosomes prior to fertilisation (Fig 1. E-G). Following fertilisation, the binding of HP1β initially occurs at the maternal centromeres, but rapidly extends to cover the entire maternal genome over the period of 1-5 hours postfertilisation (hpf). In contrast, the incoming paternal genome shows neither meH3 or HP1β binding at these early stages (1-5 hpf), although it is important to note that the paternal genome is well coated with histones at this time (Fig. 2, A-C). The absence of this heterochromatic factor on the paternal genome is therefore significant and consistent with the preferential paternal DNA demethylation event which occurs within 4 hours of fertilisation (Santos *et al.*, 2001).

The rapid and preferential recruitment of HP1β onto histone-methylated maternal chromatin at 1-5 hpf is likely to have two important consequences. First, it would protect the maternal genome from DNA demethylation, which is observed only in the paternal genome during this interval (Santos *et al.*, 2001). Second, there is growing evidence that HP1 proteins can associate with DNA methyltransferase activity (Bachman *et al.*, 2001), as well as for a direct genetic link between histone methylation and DNA methylation pathways (Tamaru and Selker, 2001). This suggests that the meH3/HP1 interactions we observe in the zygote could be linked to the

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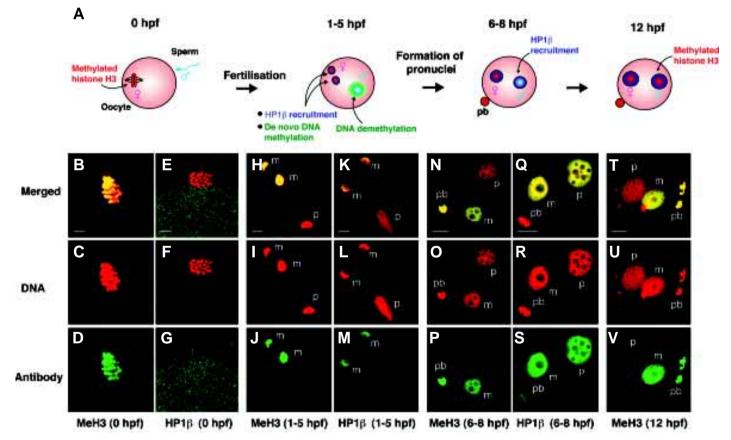
preferential *de novo* DNA methylation of the maternal genome. The methylation of the maternal genome after fertilisation has been reported previously at a number of loci in the mouse (Oswald *et al.*, 2000), and at the imprinted human *SNRPN* locus (El-Maarri *et al.*, 2001). If this is so, it is therefore also possible that a complex involving methylated histones and HP1 proteins may also contribute to the initiation of maternal imprints earlier in the developing oocyte. Indeed, the majority of the DNA methylation marks associated with imprinted genes are maternal in origin (Reik and Walter, 2001). Maternally inherited modifiers in the oocyte cytoplasm have also long been known to interact with specific loci causing aberrant *de novo* DNA methylation (Pickard *et al.*, 2001), which may also involve the meH3/HP1 interaction we have described here.

During the next phase of zygote development, the pronuclear membranes form at approximately 5-6 hpf, sequestering the parental genomes from the cytoplasm (Fig. 1A, 6-8 hpf). At this time, we first detect HP1β in the paternal pronucleus (Fig. 1, Q-S), but not meH3 (Fig. 1, N-P). Faint MeH3 staining was finally detected on the paternal genome at 12 hpf (Fig. 1, T-V). Double-staining with both meH3 and HP1β antibodies confirmed the presence of HP1β in the paternal pronucleus in the absence of meH3 (Fig 2. G-I). That this difference in meH3 staining on the parental genomes was not due to differential accessibility of the antibody was confirmed by immunostaining with a pan-histone antibody, revealing staining of equal intensity on both parental genomes (Fig 2. D-F). The recruitment of HP1β to the paternal pronucleus independently and prior to meH3 is surprising and suggests that it may be targeted via a novel mechanism. Further investigation of the precise properties of HP1β will reveal how this targeting may take place.

We also find that HP1γ is not recruited to the parental genomes at early stages after fertilisation, although it is detected in the oocyte cytoplasm by western blotting (data not shown). Instead, it is recruited equally to both parental genomes from 6-8 hpf onwards, (Fig. 3, A-I). This indicates that these two chromodomain proteins have distinct biological functions and that only HP1β is involved in the modulating epigenetic asymmetry between the parental genomes. Another consequence of pronuclear membrane formation is that the entry of proteins into the pronuclei can be regulated. For example,

Fig. 1. Epigenetic events in the mouse zygote. (A) *Schematic representation of events between 0-12 h post fertilisation (hpf). The oocyte chromosomes have a high level of methylated histone H3 (meH3) prior to fertilisation (0 hpf). At 1-5 hpf, HP1β is rapidly recruited to the two haploid maternal genomes, at the time when the paternal genome undergoes active DNA demethylation. The meH3-HP1β complex associated with the maternal genome may protect it from demethylation. It is also possible that* de novo *DNA methylation activity is targeted to the maternal genome by this heterochromatic complex. After the formation of pronuclei, HP1β is first recruited to the paternal genome (6-8 hpf) followed by meH3 at 12 hpf, restoring an equilibrium between the parental genomes. (B-P) Distribution of methylated histone H3 (meH3) and HP1β detected by immunofluorescence. (B-D) MeH3 is detected on arrested oocyte chromosomes while HP1β cannot be detected (E-G). MeH3 is restricted to the two haploid maternal genomes (m) but undetectable on paternal (p) chromatin at 1-5 hpf (H-J). At this time HP1β is recruited only to maternal (m) and not paternal (p) chromatin (K-M). With the extrusion of the polar body (pb), HP1β is lost from this maternal genome. After the formation of pronuclei, no significant meH3 can be detected on the paternal genome (N-P) although HP1β is detected here for the first time at 6-8 hpf (Q-S). MeH3 begins to be detected on the paternal genome by 12 hpf (T-V). DNA was stained with propidium iodide. Scale bars represent 10 μm (A-M) or 25 μm (N-V).*
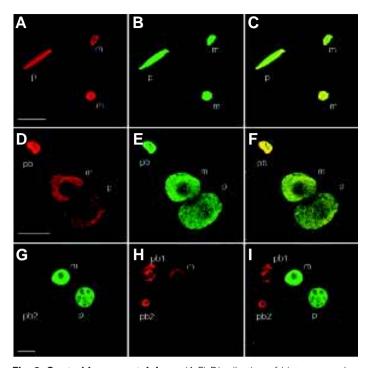
Fig. 2. Control immunostainings. *(A-F) Distribution of histones on the maternal and paternal genomes at 1-5 hpf* **(A-C)** *and 6-8 hpf* **(D-F)**. *DNA is stained with propidium iodide. Note that histones can be detected with equal intensity on both the maternal (m) and paternal (p) genomes at both timepoints. Scale bars represent 25 μm. (G-I) Double immunostaining confirming that HP1β is recruited to the paternal (p) genome at 6-8 hpf* **(G)** *in the absence of underlying meH3* **(H)**. *The merged image is shown in* **(I)**. *Scale bars represent 25 μm.*

Dnmt1 is restricted to the cytoplasm at the pronuclear stage, precluding further DNA methylation from occurring (Carlson *et al.*, 1992). Indeed, through subsequent preimplantation development the embryonic genome is subject to progressive demethylation, presumably due to a lack of maintenance methyltransferase activity, although the transient entry of Dnmt1 into the nuclei at the 8 cell stage has a key role in imprint maintenance (Howell *et al.*, 2001).

It is also interesting to note that although HP1β is recruited to centromeric foci in certain somatic cell types (Remboutsika *et al.*, 1999), we detect antibody staining throughout the parental pronuclei, albeit slightly concentrated at the borders of the prenucleolar bodies. When the localisation of centromeric DNA sequences was investigated in early embryos by three-dimensional fluorescence *in situ* hybridisation, the centromeres were found to be organised in spherical structures around the prenucleolar bodies (Fig. 3 J,K), corresponding to the slightly higher levels of HP1β staining in these regions. Staining with an antibody which specifically recognises clustered (i.e. centromeric) lysine-9 methylated histone H3 showed

Fig. 3. Distribution of HP1γ and centromeric satellite DNA in the early mouse embryo. *(A-I) Distribution of HP1γ. No significant HP1γ staining can be detected on any parental genome at 1-5 hpf* **(A-C)**. *Faint staining becomes apparent on both parental genomes from 6-8 hpf* **(D-F)**, *becoming stronger by 12 hpf* **(G-I)**. *Scale bars represent 25 μm. (J,K) Distribution of centromeric satellite DNA in 6-8 hpf* **(J)** *and 12 hpf* **(K)** *zygotes as determined by 3-dimensional FISH. The dotted lines indicate the outer limits of the pronuclei. Scale bars represent 25 μm.*

good correlation with these centromeric rings (data not shown). HP1β distribution on the maternal genome is therefore not restricted to the centromeric heterochromatin.

In view of our observations, it is of interest that the paternal pronucleus displays a much higher transcriptional competency than the maternal pronucleus in the zygote, as demonstrated by injection of reporter plasmids into either pronucleus (Aoki *et al.*, 1997). This is prior to establishment of a more generally repressive chromatin state on the paternal genome, concurrent with the first S-phase (Rastelli *et al.*, 2001). The repressive state can be reconstituted in the paternal pronucleus by the co-injection of unacetylated histones with the reporter plasmid (Rastelli *et al.*, 2001). Although the methylation status of the histones used in this experiment is unknown, it should be noted that histone H3 lysine 9 methylation correlates inversely with acetylation at this residue (Jenuwein and Allis, 2001). The establishment of the paternal repressive state might correlate with the appearance of meH3 on the paternal genome at approximately 12 hpf, the time at which S-phase commences. Studies investigating histone acetylation status in the zygote have shown that acetylation of histone H4 lysine-5 rises rapidly on that paternal genome following fertilisation while the arrested maternal chromosomes are hypoacetylated at this residue (Adenot *et al.*, 1997). It is not possible to fully correlate the histone methylation changes described in this work with the acetylation changes described by Adenot *et al.* as they refer to distinct histones. It is possible, however, to infer a broadly inverse correlation between H4 lysine-5 acetylation and H3 lysine-9
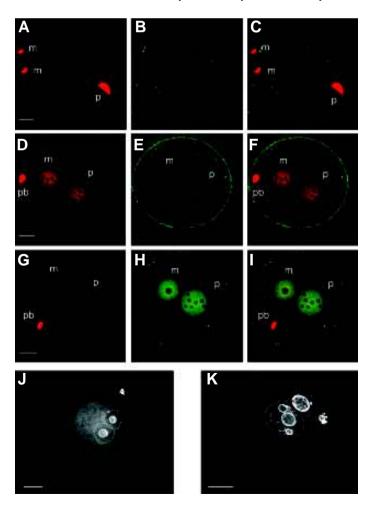
methylation in the early zygote. This reinforces the suggestion that histone modifications, including the methylation changes described here, are of prime importance in regulating the epigenetic states in the zygote. A full investigation of the interplay between various histone modifications of the parental genomes, including acetylation, methylation and phosphorylation, will be required before the roles of these modifications are fully understood in the zygote.

Experimental Procedures

Antibodies

The mouse monoclonal antibodies raised against HP1β and γ were a generous gift of P. Chambon (Remboutsika *et al.*, 1999). Lysine-9 methylated histone H3 was detected using an affinity purified rabbit polyclonal antibody (Nielsen *et al.*, 2001) and with a commercially available antibody (Upstate Biotechnology, NY). The "branched" antibody against centromeric lysine-9 methylated histone H3 was a generous gift of T. Jenuwein. The pan-histone mouse monoclonal antibody was purchased from Chemicon. Secondary anti-mouse or anti-rabbit antibodies were conjugated to Alexa™ 488 (Molecular probes)

Immunofluorescence

Embryos and unfertilised eggs were obtained from superovulated five- to six- week old female C57BL/6J x CBA/Ca mice according to standard techniques. Oocytes and zygotes were removed at an appropriate time post-hCG injection, and timing post-fertilisation judged by nuclear morphology. Most staining experiments were repeated at least twice, with over 15 embryos examined in each case for normal embryos. For embryos generated by somatic cell nuclear transfer, between 4 and 9 embryos were examined in each case. Embryos were fixed in 4 % paraformaldehyde in PBS for 15 minutes at room temperature, followed by three 5 minute washes in PBS and blocking and permeabilisation in 10 mg/ml BSA, 0.1 % Triton X-100 in PBS (BSA-Tx) for 30 minutes at room temperature. Primary antibody incubations were carried out in BSA-Tx overnight at 4°C, followed by three BSA-Tx washes of at least 15 minutes. Secondary antibodies were incubated for 1-2 hours in BSA-Tx at room temperature, followed by BSA-Tx washes as before. Embryos were then incubated in 0.1 mg/ml RNase A (Roche) in PBS at 37°C for 30 minutes. Embryos were mounted on slides in Vectashield (Vector Laboratories) containing DAPI and propidium iodide. Primary antibodies were diluted 1:200 while secondary antibodies were diluted 1:500.

3-Dimensional Fluorescence **In Situ** *Hybridisation (FISH)*

Embryos were washed in acid Tyrode's solution to remove the zona pellucida and washed in T6 medium with 6 mg/ml Polyvinylpyrrollidone (Sigma). Embryos were stuck to coverslips pretreated with 0.2 mg/ml concanavalin A by centrifugation. Cells were treated for three-dimensional FISH analysis essentially as described in Brown *et al.*, 1997, omitting the EGS crosslinking step. The gamma satellite (centromeric) probe was a generous gift of N. Dillon, and was labelled using a standard nick translation protocol with Fluorored (Amersham Pharmacia Biotech).

Confocal Microscopy

Images were captured with a Nikon Eclipse E800 confocal scanning laser microscope, using Biorad Lasersharp software. Serial optical sections (Z-series) were collected at 0.5 μm intervals encompassing all the nuclear entities present. These Z-series were stacked to produce an image depicting staining patterns and intensities of all the chromosome sets present.

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