Maturation of mouse fetal germ cells *in vitro*

Even immature oocytes can eventually be fertilized after some skilful manipulation.

Nuclear reprogramming is essential during gametogenesis for the production of totipotent zygotes. Here we show that premeiotic female germ cells derived from mouse fetuses as early as 12.5 days post coitum are able to complete meiosis and genomic imprinting *in vitro* and that these matured oocytes are highly competent in supporting development to full term after nuclear transfer and *in vitro* fertilization. To our knowledge, this is the first time that complete oogenesis has been successfully accomplished *in vitro*.

Although the ovaries of mammals contain thousands or millions of immature oocytes, few of these ever mature to the point at which reproduction *in vivo* is possible. Ovarian oocytes therefore constitute a large and potentially valuable resource for clinical and zoological application. However, although mature oocytes have been produced *in vitro* by culturing immature oocytes[1–3], oogenesis was never complete, and attempts to produce even non-growing oocytes at the diplotene stage of the first meiosis from ovaries derived from newborn mice have met with limited success (only 0.016%; ref. 2).

We have shown that this poor ability of oocytes to mature in culture is due to their incompetent cytoplasm[4], a problem that can be overcome by transferring their nuclei into enucleated, fully grown oocytes. Although live pups can eventually be produced from oocytes reconstituted in this way, this is not possible if nuclei from small, immature oocytes are used for reconstitution, probably because of defects in their meiotic chromosomal configuration and/or genomic imprinting[5–7].

We cultured premeiotic female germ cells *in vitro* in an attempt to complete this essential nuclear reprogramming (see supplementary information for details). Ovaries from mouse fetuses at 12.5 days post coitum (d.p.c.), with the mesonephroi attached, were cultured for 7 days, followed by removal of the mesonephroi and a further 10 days in culture (Fig. 1). These cultured ovaries contained many secondary follicles, which we isolated and cultured for 11 days; at the end of the 28-day culture period, some follicles showed antrum formation and the oocytes had increased in diameter (63.9 μm, $n = 127$; Fig. 1).

We estimated the extent of genomic imprinting in these oocytes by analysing DNA methylation in the imprinted gene *Igf2r* (ref. 8). The methylation pattern at each stage of culture was consistent with that in oocytes taken from mice at the same stage (see supplementary information), indicating that normal imprinting can be established *in vitro*.

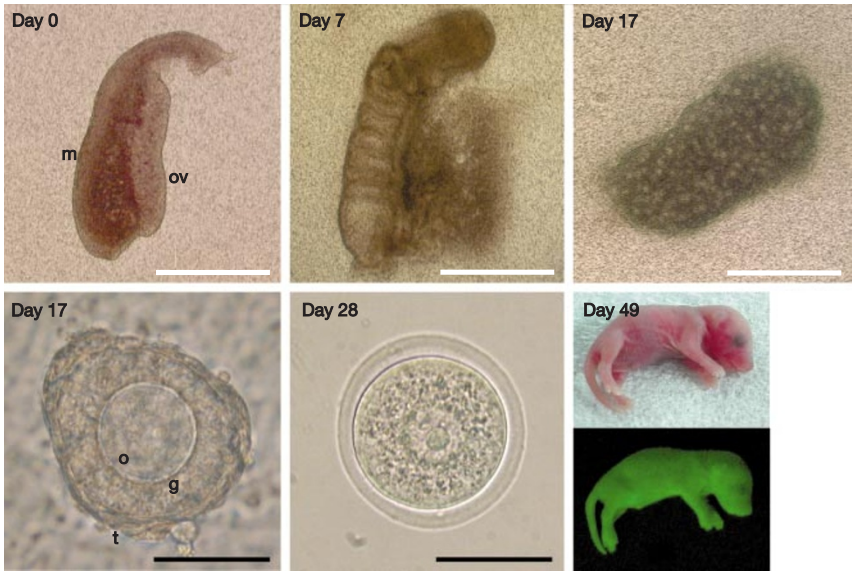

Figure 1 Development of premeiotic female germ cells *in vitro*. Day 0, ovary (ov) from a green-fluorescent-protein (GFP)-labelled B6CBF1 (GFP–C57BL/6J × CBA) transgenic mouse fetus at 12.5 d.p.c.[11], with attached mesonephroi (m). Ovaries were cultured in Waymouth medium supplemented with 10% fetal bovine serum (FBS) on a Costar Transwell membrane[2]. At day 7 the mesonephroi was removed and at day 17 a secondary follicle, consisting of theca cells (t) and 2–3 layers of granulosa cells (g) around the oocyte (o), was isolated from the ovary and cultured in MEM-α medium supplemented with 5% FBS, 0.1 IU ml^{-1} follicle-stimulating hormone, 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin and 5 ng ml^{-1} selenium on a Costar Transwell-COL membrane[2,3]. Day 28, oocyte isolated from cultured follicle for nuclear transfer. After nuclear transfer and *in vitro* fertilization, the resulting zygotes (bottom right. day 49) were cultured in M16 medium. All cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in air. Scale bars: white, 500 μm; black, 50 μm.

We also investigated whether nuclear reprogramming could occur in these oocytes by monitoring their development (see supplementary information). Because oocytes isolated from cultured follicles were unable to resume meiosis (0/38, 0%), we transferred the nuclei into enucleated, fully grown oocytes from adult mice. These reconstituted oocytes were able to resume meiosis and mature into metaphase in the second meiosis (M II, 101/108, 94%) and had a normal karyotype ($n = 20$, 11/11, 100%), indicating that chromosomal maturation for meiosis can be completed *in vitro*. As expected[4], oocytes reconstituted with intact female germ cells from fetuses at 12.5 d.p.c. became aneuploid (32/32, 100%) owing to abnormal chromosomal segregation.

As oocytes reconstituted by a single nuclear transfer did not develop efficiently into blastocysts (14/34, 41%), we used serial nuclear transfer (see supplementary information for details). The rate of *in vitro* fertilization of these reconstituted oocytes was normal (72/81, 89%) and they developed efficiently into blastocysts (64/72, 89%).

After embryo transfer to a surrogate mother, 16 living pups were obtained from 7 mothers at 19.0 d.p.c. by caesarean section (16/64, 25%). No obvious abnormality was seen in any of the pups (*in vitro* pup weight compared with that *in vivo*: 1.46 g versus 1.34 g, $P > 0.1$) or placentae (*in vitro* weight compared with that *in vivo*: 133 mg versus 126 mg, $P > 0.5$), as expected from normal imprinted methylation of *Igf2r*, *Snrpn* and *Peg1* (see supplementary information)[9,10]. These animals were fertile after puberty.

We have shown that the most primitive murine fetal oocytes can differentiate into competent oocytes with high efficiency. As well as offering an opportunity to analyse the mechanisms behind nuclear reprogramming *in vitro*, our system might eventually help women undergoing chemotherapy or radiotherapy to become mothers afterwards, by prior removal of an ovary.

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Gene silencing

Trans-histone regulatory pathway in chromatin

The fundamental unit of eukaryotic chromatin, the nucleosome, consists of genomic DNA wrapped around the conserved histone proteins H3, H2B, H2A and H4, all of which are variously modified at their amino- and carboxy-terminal tails to influence the dynamics of chromatin structure and function[1,2] — for example, conjugation of histone H2B with ubiquitin controls the outcome of methylation at a specific lysine residue (Lys 4) on histone H3, which regulates gene silencing in the yeast *Saccharomyces cerevisiae*[3]. Here we show that ubiquitination of H2B is also necessary for the methylation of Lys 79 in H3, the only modification known to occur away from the histone tails, but that not all methylated lysines in H3 are regulated by this '*trans*-histone' pathway because the methylation of Lys 36 in H3 is unaffected. Given that gene silencing is regulated by the methylation of Lys 4 and Lys 79 in histone H3, we suggest that H2B ubiquitination acts as a master switch that controls the site-selective histone methylation patterns responsible for this silencing.

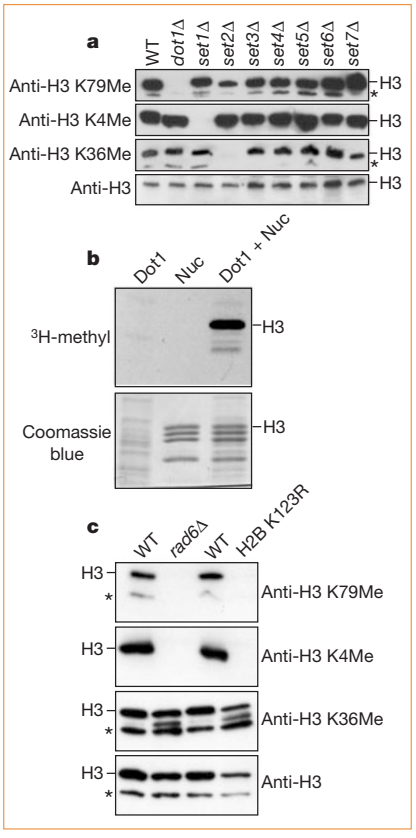

Figure 1 Regulation of Lys-79 methylation on histone H3. **a,** Western blot of nuclear extracts isolated from mutant yeast strains carrying the indicated deletions and probed with antibodies against the methylated H3 lysine residues K79Me, K4Me or K36Me. **b,** Polyacrylamide-gel electrophoresis analysis of recombinant Dot1 for histone methyltransferase activity on nucleosomal substrates (Nuc) *in vitro*, as revealed by autoradiography (top) and Coomassie-blue staining of histones (bottom). **c,** Western blot of nuclear extracts from wild-type yeast and *rad6*-deleted or H2B K123R mutant strains probed with the histone antibodies indicated. Asterisks, an H3 proteolysis product that removes the methylation site at Lys 4 but not at Lys 36 or Lys 79.

Lysine residues subject to methylation in yeast histone H3 are Lys 4 and Lys 36 near the amino terminus, and Lys 79 (refs 4–6, and data not shown), a modification site that is unique in that it is located away from the H3 tails in the first loop of the histone-fold domain. To identify what mediates methylation of Lys 79, we used an antibody raised against this methylated site (anti-H3 K79Me; Upstate Biotechnology) to screen nuclear extracts isolated from yeast strains containing deletions of known and putative histone methyltransferase enzymes.

We identified Dot1, a factor involved in gene silencing[4,5], as a gene product that is essential for methylation of Lys 79 (Fig. 1a), in agreement with earlier findings[4–6]. This was unexpected, as Dot1 lacks the 'SET' domain, which until now was thought to be the only domain responsible for methylating histone lysine residues. No other protein containing the SET domain was found to mediate Lys-79 methylation (Fig. 1a). To confirm that Dot1 is the enzyme responsible, we showed that expression of *DOT1* in a *dot1*-deleted strain restores Lys-79 methylation in H3 (see supplementary information) and that recombinant Dot1 contained methyltransferase activity towards nucleosomal H3 (Fig. 1b). Dot1, which resembles the arginine methyltransferase family in sequence and structure[7], therefore represents a new class of lysine-specific histone methyltransferase enzymes.

Ubiquitination of histone H2B is mediated by the enzyme Rad6, also known as ubiquitin-conjugating enzyme Ubc2 (ref. 8). We tested whether Rad6 ubiquitination of H2B could influence methylation at H3 sites apart from Lys 4 (ref. 3). Surprisingly, we found that there was a loss of Lys-79 methylation, but not of Lys-36 methylation, in strains deleted for *RAD6* or mutated at the H2B-ubiquitination site (K123R; Fig. 1c). We verified that Dot1 is expressed in these mutant strains by using reverse transcription followed by polymerase chain reaction to detect the presence of its messenger RNA (see supplementary information). We conclude that Rad6 ubiquitination of H2B at Lys 123 specifically regulates the methylation of both Lys 4 and Lys 79 of H3 in a '*trans*-histone' pathway.

We found that deletion of *DOT1*, *SET1* (refs 9, 10) or *SET2* (ref. 11) results in the specific loss of their respective modifications (Fig. 1a), indicating that the absence of these individual modifications does not affect the others. Given that the regulation of Lys-4 methylation by H2B ubiquitination is unidirectional[3] and that Lys-4 methylation still occurs in the *dot1*-deleted (Fig. 1a) and Lys-79 mutant strains (see supplementary information), we conclude that the regulation of Lys-79 methylation by H2B ubiquitination is also unidirectional.

Our findings indicate that methylation of histone H3 at Lys 4 and Lys 79, but not at Lys 36, is regulated by the ubiquitination of histone H2B. As Lys 4 and Lys 79 methylation both mediate gene silencing, we propose that H2B ubiquitination acts as a master switch of gene silencing through a *trans*-histone pathway that leads to the appropriate patterns of histone methylation. Given that some lysine residues in H3 are affected by this pathway, but not others, our results lend support to the 'histone code' hypothesis[2].

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