Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse

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In mammals, the maternal and paternal genomes are required for embryonic development[1–5]. This is due to genomic imprinting which leads to the expression or repression of genes solely on the basis of the parent from which they were inherited[6–9]. As a result, parthenogenetic embryos die before day 10 of gestation and show limited development of extra-embryonic membranes[3,4]. Maternal imprinting is established during oogenesis[6–11] and is associated with allele specific modifications in DNA methylation. We have investigated epigenetic modifications during oocyte growth using nuclear transfer techniques to produce mature oocytes with maternal chromatin derived from non-growing oocytes. Parthenogenetic activation of such oocytes leads to the development of normal size fetuses with a well developed placenta on day 13.5 of gestation; three days further than previously recorded for parthenogenetic development. In contrast, after fertilization, only one embryo was recovered on 9.5 days of gestation. Further, in these embryos we investigated the well characterized methylation pattern of the maternally expressed insulin-like growth factor II receptor gene (*Igf2r*) and found that the pattern of methylation was indeed different to that of fertilized control embryos. Thus, the embryonic phenotypes observed here correlate with changes in epigenetic events that normally occur during oocyte growth.

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To investigate the effect of epigenetic changes during oocyte growth on embryogenesis we developed a technique for producing embryos containing one set of chromosomes arising from non-growing oocytes (see Fig. 1). This was performed by fusing a non-growing primary oocyte (ng) from a one-day-old mouse with a fully grown oocyte (fg) from which the germinal vesicle had previously been removed (Experimental group; Fig. 1). Controls were produced by the fusion of an enucleated fully grown oocyte with a germinal vesicle isolated from a different fully grown oocyte. Subsequently the experimental and control groups were exposed to the same manipulations (Fig. 1). The two groups of oocytes were matured *in vitro*. Approximately 80% of the oocytes in both groups arrested at metaphase II (MII) but on fertilization in vitro the experimental oocytes failed to form pronuclei. To determine if this inhibition was due to the absence of pronuclear forming factor[12], thought to be present in the germinal vesicle, the MII chromosomes from both groups were transferred into enucleated or intact ovulated MII oocytes (Fig. 1). On fertilization or parthenogenetic activation such oocytes were able to support normal pronuclear formation suggesting that factors necessary for this event accumulate in the germinal vesicle (GV) during oocyte growth. The different experimental and control oocytes and embryos produced are identified according to the origin of the chromatin (see Fig. 1).

The production of embryos with maternal alleles that have not been subject to maternal epigenetic modifications during oocyte growth provides the first opportunity to investigate the role of these modifications in embryonic development (Table 1; Figs 2, 3). The levels of fertilization and parthenogenetic activation were similar in all of the groups (Table 1). Development to the blastocyst stage and the rates of implantation after embryo transfer was normal in embryos containing at least one set of alleles from fully grown oocytes or sperm. However, in embryos with maternal alleles derived entirely from small non-growing oocytes ($MII^{ng/ng}$), only 13% formed 8-cell embryos (Table 1). These findings suggest that at least some epigenetic modifications are required on either the maternal or paternal alleles for embryos to progress beyond the 8-cell stage.

The phenotype of embryos after implantation was dramatically influenced by the presence of the unmodified maternal alleles and was dependent on whether the complementary set of alleles were paternal or maternal in origin. Development of fertilized $MII^{ng/sp}$ (see Fig. 1 legend) embryos was restricted such that at 9.5 days post coitum (dpc) only one stage 11 (8 dpc)[13] embryo was recovered (Table 1; Fig. 3*e*). In contrast parthenogenetic $MII^{ng/fg}$ embryos developed to day 13.5 dpc, at least 3.5 days further than has been previously reported for parthenogenesis[1–5]. These conceptuses were generally well developed and contained live fetuses estimated to be at stage 21 (13.5 dpc)[13] (Fig. 3*a,b*). Similar fetuses were recovered on 14.5 and 15.5 dpc but were no longer alive (Fig 3*c,d*). In some fetuses the crown-rump length reached >10 mm, the digits of the hand plate had separated and the heart was beating. Unlike parthenogenetic conceptuses described in previous studies[1–5] the placenta was extensively developed (see Fig. 3*a*). Development of control $MII^{fg/sp}$ embryos was normal, with the birth of live young, while control $MII^{fg/fg}$ parthenogenetic embryos implanted, but died soon afterwards (Table1). These results suggest that epigenetic modifications during oocyte growth are important in the regulation of embryonic development.

A useful marker of epigenetic modifications is allele specific patterns of DNA methylation. Some allele specific patterns of methylation are established during oogenesis[7,10,11,14–16], but only one of these is stable throughout early development; that is the methylation of the intronic site 3 of region 2 of the maternally expressed *Igf2r* allele[7,11]. Initially we confirmed previous findings that the locus is unmethylated in non-growing primary oocytes and methylated in mature oocytes[7,11] (Fig. 4). In MII^{ng} oocytes and blastocysts resulting from the fertilization of these oocytes the locus was indeed unmethylated (Fig. 4), confirming that epigenetic differences exist, depending on the origin of the maternal chromatin. These findings support

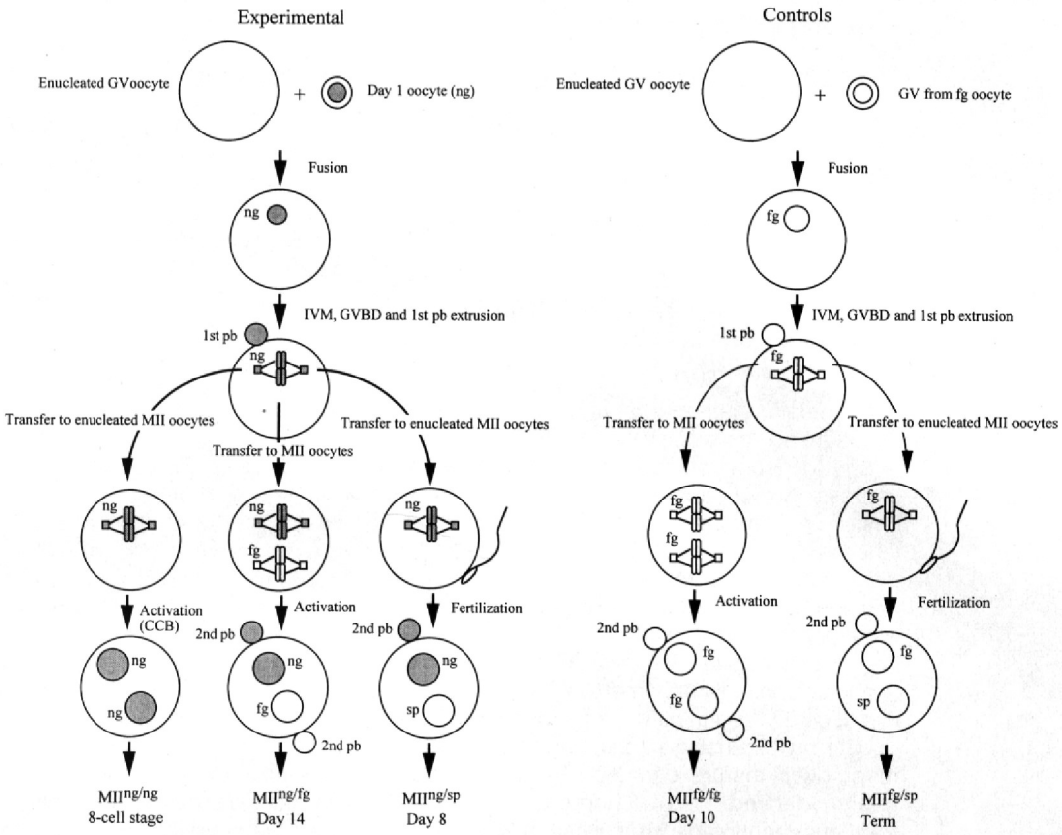

Fig. 1 The production of oocytes containing maternal alleles from different sources and the developmental outcome of resulting embryos. Oocytes containing alleles from non growing oocytes (ng) were produced by fusing an oocyte from a one-day-old mouse with an enucleated fully grown oocyte. Oocytes and embryos containing chromatin from non-growing oocytes are referred to as experimental. Control oocytes were produced by fusing an enucleated oocyte with an isolated germinal vesicle (GV) from a fully grown (fg) oocyte. The oocytes from both groups were matured *in vitro* (IVM) and the metaphase chromosomes from the metaphase II (MII) arrested oocytes were transferred to intact or enucleated ovulated oocytes. The oocytes were then parthenogenetically activated in the presence and absence of cytocholasin B (CCB) or fertilized *in vitro*. Thus three types of experimental embryos with maternal chromatin from a variety of sources were produced. First, oocytes with maternal chromatin from non growing oocytes (MII^{ng}) were activated in the presence of cytocholasin B to produce diploid parthenogenetic embryos where the entire chromatin complement was derived from small non-growing oocytes ($MII^{ng/ng}$). Second, embryos were produced by the parthenogenetic activation of $MII^{ng/fg}$ oocytes leading to an embryo with one set of homologous maternal chromatin and another that is derived from a small non-growing oocyte ($MII^{ng/fg}$). Third, MII^{ng} oocytes were fertilized *in vitro* to produce embryos with maternal chromatin complement from small oocytes and a normal paternal chromatin complement ($MII^{ng/sp}$). Control oocytes were either parthenogenetically activated ($MII^{fg/fg}$) or fertilized *in vitro* ($MII^{fg/sp}$).

the idea that the phenotype of $MII^{ng/ng}$, $MII^{ng/fg}$ and $MII^{ng/sp}$ embryos is a result of epigenetic modifications that occur during oocyte growth.

The reason that epigenetic differences modify embryonic phenotype is presumably by altering the pattern of gene expression during embryogenesis. The molecular basis of allele specific gene expression is unknown[17,18], but disruption of methylation can lead to biallelic expression of imprinted genes[19]. Changing the normal pattern of methylation, as we have done

Table 1 Development of embryos containing chromatin from non-growing primary oocytes

Type of reconstituted oocyte	Normally fertilized or activated oocytes/ total	Blastocysts/ total	Pregnant recipients/ total	Day of autopsy	Conceptuses/ total	Fetuses			
						<8mm	>8mm	>9mm	>10mm
Experimental									
MII ng/ng (parthenogenetic)	39/43	0/39 (5/39 8-cells)							
MII ng/sp (fertilized)	122/199	70/101	1/1	9.5 dpc	6/11	1			
			1/1	10.5 dpc	6/11				
			1/1	11.5 dpc	9/10				
			2/3	12.5 dpc	13/16				
MII ng/fg (parthenogenetic)	175/274	124/131	2/2	13.5 dpc	13/18		2	3 (2 alive)	1 (alive)
			2/2	14.5 dpc	10/15	1	1	2	1
			2/2	15.5 dpc	14/18	3	3	1	1
Controls									
MII fg/sp (fertilized)	16/31	11/16	2/2	19.5 dpc	6/11	4 live young			
MII fg/fg (parthenogenetic)	32/50	27/32	2/2	11.5 dpc	15/27	all resorbed			

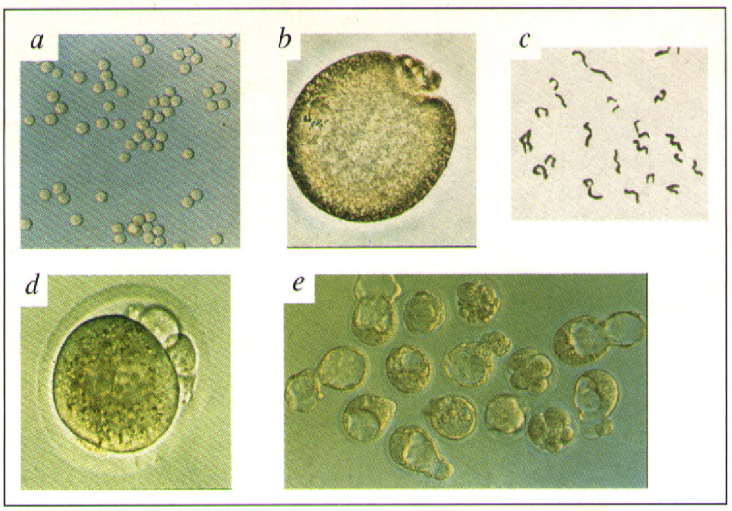

Fig. 2 Production of oocytes and embryos containing the nucleus of non-growing primary oocytes. *a*, Non-growing primary oocytes isolated from a 1-day-old female mouse. *b*, An experimental oocyte containing chromatin from a nongrowing oocyte (MII^{ng}). *c*, The bivalent chromosomes of an MII^{ng} experimental oocyte. *d*, A pronucleate stage parthenogenetic $MII^{ng/fg}$ embryo derived from an experimental oocyte. *e*, Blastocysts obtained after culture of embryos shown in (*d*) for 4 days.

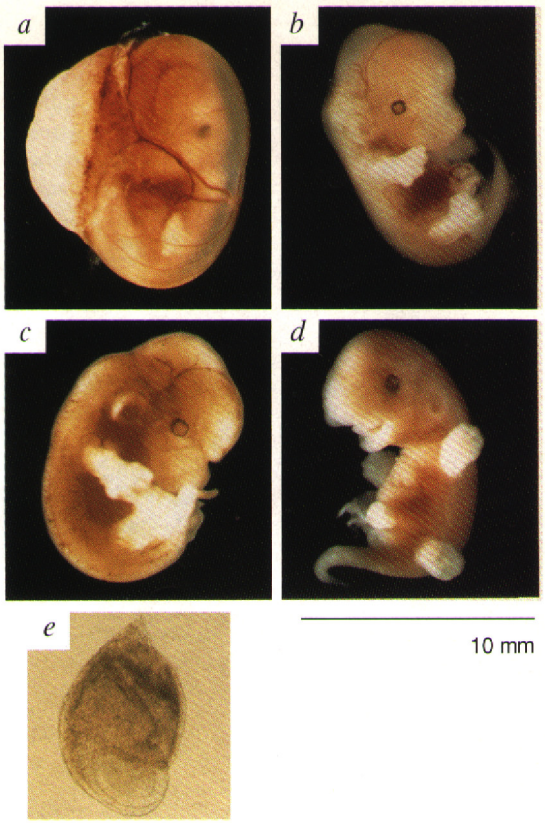

Fig. 3 Post-implantation development of embryos containing maternal alleles from non-growing oocytes after fertilization or activation. *a*, A live parthenogenetic $MII^{ng/fg}$ embryo at 13.5 dpc. Note the extensive development of the placenta and extra-embryonic membranes. *b*, The fetus after removal of extra-embryonic tissues. *c*, *d*, Similar fetuses from parthenogenetic $MII^{ng/fg}$ conspectuses on 14.5 and 15.5 dpc, respectively. These fetuses were dead at the time of recovery. *e*, The single $MII^{ng/sp}$ conceptus recovered on day 9.5 of gestation.

here for the *Igf2r* may, therefore, be expected to modify the expression of this and other imprinted genes. Although quantification of allele-specific gene expression[18,20] is required to confirm this possibility, the developmental phenotype seen in this study is consistent with the effects of imprinted genes on parthenogenetic development.

The small size of parthenogenetic embryos and the growth retardation seen in parthenogenetic chimaeras[3–5] suggests that maternally expressed genes generally suppress growth. Thus lack of expression of these genes from the maternal alleles derived from non-growing oocytes may explain the increased embryonic and placental development compared to standard parthenogenones. *Igf2r* is a good candidate for such a role since the *Igf2r* null phenotype is a 25–30% increase in body size[21]. Other genes may be important specifically for placental development, such as the maternally expressed *Mash2* (ref. 22), and paternally expressed (maternally repressed) genes necessary for proliferation of the polar trophectoderm cells[23]. Failure to suppress the expression of such genes from alleles from non-growing oocytes would also increase placental development.

The stability of the modified pattern of methylation of the *Igf2r* locus in oocytes and embryos containing maternal alleles from non growing oocytes has implications for imprinting mechanisms. The failure to correct the pattern of methylation during oocyte maturation and development to the blastocyst stage suggests that the mechanism responsible for methylating this site is functional only during a spe-

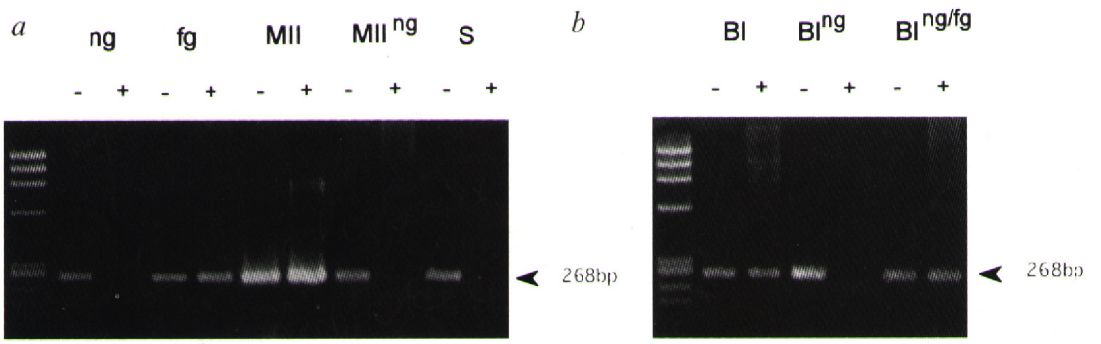

Fig. 4 Analysis of the pattern of DNA methylation of the *Igf2r* gene in reconstituted oocytes and embryos. *a*, Methylation patterns in intact and experimental gametes. Non-growing primary oocytes from 1-day-old mice (ng), fully grown GV stage oocytes (fg), ovulated MII oocytes (MII), experimental oocytes containing chromatin from a non growing oocyte (MII^{ng}), and epididymal sperm (S). *b*, Methylation patterns in blastocysts derived from these gametes. Normal blastocysts from intact MII oocytes (BI), blastocysts derived from the fertilization of MII^{ng} oocytes (BI^{ng}), blastocysts derived from parthenogenetically activated $MII^{ng/fg}$ oocytes ($BI^{ng/fg}$). Lanes (–) and (+) are *Hpa*II digested and undigested samples, respectively.

cific stage of oogenesis, before the resumption of meiosis. The restricted period for epigenetic changes such as methylation and those required for development may be due to modifications in chromatin structure during oocyte growth or the transient presence of necessary oocyte specific factors. Our preliminary experiments show that alleles from growing oocytes obtained from 13-day-old mice do not support full term development suggesting that the epigenetic modifications required for development occur late in oogenesis, during the second half of the growth phase.

In conclusion, we show that epigenetic modifications during oocyte growth have dramatic consequences for subsequent development after fertilization or parthenogenetic activation. The ability to identify precisely when, during oocyte growth, genomic imprinting takes place provides an exciting prospect for investigating the molecular mechanism of genomic imprinting in the gametes.

Methods

Production of reconstituted embryos. B6CBF1 (C57BL/6j × CBA) mice were used as oocyte and sperm donors. Fully grown GV stage oocytes were collected from ovarian follicles 44–48 h after injection of PMSG. Non-growing primary oocytes were obtained from ovaries of one-day-old mice[24]. Nuclear transfer was carried out after removing the cumulus cells from the fully grown oocytes as described[25,26]. Fusion of non growing primary oocytes with enucleated GV oocytes was induced with inactivated Sendai virus (HVJ, 2700 haemagglutonating activity U/ml). The GV stage oocytes were manipulated in a medium containing 200 μM dbcAMP and 5% calf serum and released from the medium 1 h after fusion. The reconstituted oocytes were cultured for 14 h in Waymouth MB752/1 (Gibco)[24]. To control for manipulations in this study, a GV isolated from a fully grown oocyte was fused as described with an enucleated fully grown oocyte. From this stage control and experimental oocytes were exposed to the same manipulations and treatments. The MII chromosomes of the experimental and control oocytes were transferred into enucleated or intact MII oocytes collected from oviducts of superovulated mice 15–16 h after hCG. The former type of oocytes was fertilized *in vitro* and the latter was activated with 10mM $SrCl_2$ in Ca^{2+} free M16 medium for 1 h (ref. 27). These embryos were manipulated in M2 medium and cultured in M16 medium[28] as described[25]. Blastocysts derived from reconstituted oocytes were transferred into the uterine horns of CD-1 female mice at 2.5 days of pseudopregnancy.

PCR analysis of *IGF2r* methylation. DNA from 100–150 germ or embryonic cells was obtained by the guanidine-HCl extraction method as described[7,11,15,16]. PCR methylation analysis of *Hpa*II sensitive site in site 3 of region 2 of the *Igf2r* was carried out as described[7,11,15]. The DNA sample was divided into two aliquots that were digested with either *Pvu*II or *Pvu*II + *HpaII*. These samples of DNA were added to PCR buffer with the appropriate primers. Each PCR contained DNA from about 30 cells.

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