Unaltered imprinting establishment of key imprinted genes in mouse oocytes after *in vitro* follicle culture under variable follicle-stimulating hormone exposure

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ABSTRACT **Imprinted genes are differentially methylated during gametogenesis to allow parental-specific monoallelic expression of genes. During mouse oogenesis, DNA methylation at imprinted genes is established during the transition from primordial to antral follicle stages. Studies in human and mouse suggest aberrant imprinting in oocytes following *in vitro* maturation and after superovulation with high doses of gonadotrophines. The exact mechanisms leading to aberrant imprinting are unknown. We examined the methylation status of differentially methylated regions of key imprinted genes (by bisulfite sequencing) in mouse metaphase II oocytes, grown in a long-term pre-antral follicle culture system and matured *in vitro*, in the presence of a physiological (10 IU/L) and a high (100 IU/L) recombinant FSH dose. Our results showed a normal DNA methylation at the studied regulatory sequences of *Snrpn*, *Igf2r* and *H19*, demonstrating that 1) prolonged culture and *in vitro* maturation do not *per se* modify the establishment of imprinting in oocytes and 2) supraphysiological FSH doses do not induce aberrant DNA methylation at the studied regulatory sequences in this system.**

KEY WORDS: *DNA methylation, follicle culture, genome imprinting, in vitro maturation, FSH, mouse oocyte*

Introduction

Genomic imprinting is a phenomenon leading to sex-specific monoallelic expression of genes (Surani *et al.*, 1984; Reik and Walter, 2001). Imprinting explains why both maternal and paternal genomes are required in normal development (McGrath and Solter, 1984). Imprinted genes play important roles in embryo development and growth, placental differentiation and neurobehavioural processes (reviewed by Isles and Holland, 2005; Fowden *et al.,* 2006; Smith *et al.,* 2006). Furthermore, aberrant imprinting is linked to human diseases like Beckwith-Wiedemann, Prader-Willi and Angelman syndromes (reviewed by Paulsen and Ferguson-Smith, 2001).

DNA methylation is considered an important epigenetic mechanism for differentially marking the parental alleles of imprinted genes, which is involved in sex-specific gene expression (reviewed by Li *et al.*, 2002). DNA methylation occurs at cytosine residues, mainly within CpG dinucleotides, and is catalyzed by a family of DNA methyltransferases (Bestor *et al.*, 2000). For imprinted genes, the gene itself or nearby regulatory sequences are differentially methylated in the parental alleles: these regions are the so-called differentially methylated regions (DMR). Imprints are erased in primordial germ cells (Hajkova *et al.*, 2002; Lee *et al.*, 2002) and reset during gametogenesis in a sex-specific manner; the latter is called primary imprinting. In mouse, primary imprinting is established at a specific time for each gene during postnatal oocyte growth (Bao *et al.*, 2000; Obata and Kono, 2002). DNA methylation occurs asynchronously at different imprinted genes, while oocytes are arrested at prophase I during the transition from primordial to antral follicle stages (Lucifero *et al.*, 2002 and 2004; Hiura *et al.*, 2006). This acquisition of DNA methylation during oogenesis correlates with an increase in oocyte diameter (Lucifero *et al.*, 2004; Hiura *et al.*, 2006).

Several studies have linked assisted reproductive technology (ART) to aberrant imprinting. *In vitro* culture of pre-implantation embryos was associated with aberrant expression and/or DNA methylation of imprinted genes in different species (reviewed by Khosla *et al.*, 2001). Notably, the addition of serum to embryo

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culture medium in mouse is associated with aberrant imprinting (Khosla *et al.*, 2001), although the exact causative factor in serum remains unknown.

Recent studies suggest that manipulation of oocytes is also associated with aberrant imprinting. A study in human suggested aberrant DNA methylation at the imprinted *H19* gene in oocytes following *in vitro* maturation (IVM) (Borghol *et al.*, 2006) and prolonged IVM of mouse oocytes was associated with a loss of DNA methylation at *Peg1* (Imamura *et al.*, 2005). Another study reported aberrant DNA methylation at imprinted genes in oocytes after superovulation with high doses of gonadotrophins in mouse and human (Sato *et al.*, 2007).

Several studies have suggested an increased incidence in rare human imprinting disorders in children conceived after ART (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003). Methylation analysis in the affected ART-children revealed a hypomethylation of the maternal allele at the imprinted *Snrpn* and *Kcnq1ot1* locus, which are involved in respectively Angelman and Beckwith-Wiedemann syndrome (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003). The exact mechanisms that lead to aberrant imprinting of these maternal alleles after ART are unknown. Therefore, more research is needed to determine causative factors for aberrant imprinting in ART protocols.

Follicle culture and IVM are new alternative techniques progressively being introduced into the assisted reproductive laboratories to obtain large numbers of oocytes for cloning or to avoid using any hormones in well-defined patient groups (Ohkoshi *et al.*, 2003; Suikkari and Soderstrom-Anttila, 2007; Holzer *et al.*, 2007). However, only very limited information is currently available on the effects of IVM on DNA methylation of imprinted genes in oocytes. A mouse follicle culture system allows the growth of massive amounts of oocytes from early pre-antral follicles up to fertilisable metaphase II (MII) oocytes under fully defined conditions during a 13-day culture period (Cortvrindt and Smitz, 2002). The first aim of the study was to compare the DNA methylation status at regulatory sequences of 4 imprinted genes in mouse MII oocytes obtained after prolonged follicle culture and *in vitro* oocyte maturation with *in vivo* grown oocytes after conventional superovulation (Cortvrindt and Smitz, 2002).

Studies on the effects of superovulation on DNA methylation have shown conflicting results. One study reported aberrant DNA methylation at the *H19* gene in oocytes after superovulation with high doses of gonadotrophins in mouse and human (Sato *et al.*, 2007). However, two recent studies have shown that superovulation may induce aberrant expression of imprinted genes in blastocysts (*H19*) or 9.5 dpc placentae (*H19* and *Snrpn*) with normal DNA methylation patterns at regulatory sequences of the studied imprinted genes (Fauque *et al.*, 2007; Fortier *et al.*, 2008). The latter studies suggest that superovulation interferes with the maintenance of imprinting after fertilization, but that DNA methylation at regions known to be important for imprint establishment in oocytes is not altered by superovulation. Therefore, the second aim of our study was to assess the influence of high doses of recombinant FSH (r-FSH) in the follicle culture system on DNA methylation of 3 imprinted genes in MII oocytes.

We analyzed differentially methylated regions of small nuclear ribonucleoprotein N (*Snrpn*), insulin-like growth factor 2 receptor (*Igf2r*), paternally expressed gene 3 (*Peg3*) and *H19* by the bisulphite sequencing technique. In mouse oocytes, the DMRs of *Snrpn*, *Igf2r* and *Peg3* acquire DNA methylation during oogenesis in the post-natal growth phase (Lucifero *et al.*, 2004). At *H19*, DNA methylation is acquired in the male germ line (Davis *et al.*, 1999 and 2000). We chose these genes because methylation dynamics during oogenesis have been extensively characterized for these genes, and because of their biological significance. The analyzed sequence for *Snrpn* corresponds to the human *SNRPN* locus, in which abnormal methylation was related to Prader-Willi and Angelman syndromes (Bielinska *et al.*, 2000). The *Igf2r* gene plays an important role in embryo development and fetal growth, and abnormal *Igf2r* imprinting was linked to large offspring syndrome after *in vitro* embryo culture in sheep (Young *et al.*; 2001). Abnormal methylation at *H19* was linked to Beckwith-Wiedemann (DeBaun *et al.*, 2003). Moreover, a gain of methylation at *H19* in oocytes
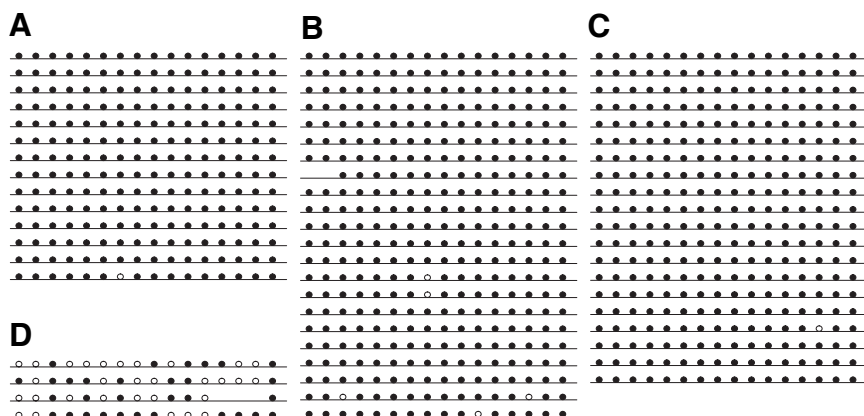
Fig. 1. DNA methylation of *Snrpn* (DMR1) (A) *in in vivo grown and PMSG/hCG superovulated MII oocytes;* **(B)** *in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 10 IU/ L r-FSH;* **(C)** *in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 100 IU/L r-FSH and* **(D)** *in oocytes from early pre-antral follicles from 13-14 day-old mice. Each line represents an individual allele and each circle represents a CpG site within the DMR analyzed. Filled circles, methylated cytosines; open circles, unmethylated cytosines; missing circles, CpG sites whose methylation status could not be determined.*

Footnote: sequence polymorphisms (outside CpG-sites) between clones with similar methylation patterns were examined to ensure that only clones representing different alleles were presented; results are derived from (A) three independent bisulphite sequencing experiments performed on 3 pools of 100-150 MII oocytes from 9 superovulated mice; (B) four independent bisulphite sequencing experiments performed on 4 pools of 100-150 MII oocytes from 4 independent *in vitro* follicle culture experiments with 10 IU/L r-FSH (involving 4 mice per culture experiment); (C) four independent bisulphite sequencing experiments performed on 4 pools of 100-150 MII oocytes from 4 independent *in vitro* follicle culture experiments with 100 IU/L r-FSH (involving 4 mice per culture experiment) and (D) one bisulphite sequencing experiment performed on one pool of 100 oocytes from early pre-antral follicles (derived from four 13-14 day-old mice).

has been described after IVM in human and after superovulation in human and mouse (Borghol *et al.*, 2006; Sato *et al.*, 2007).

Results

DNA methylation patterns at imprinted genes in cumulus cells

PCR amplification on bisulphite-treated DNA is prone to PCR bias, most commonly resulting in preferential amplification of DNA stands that are unmethylated at CpG-sites (Warnecke *et al.*, 1997). Therefore, the DNA methylation analysis was validated on pools of approximately 100-200 cumulus cells.

One or two PCR amplifications were performed for each gene and 8 clones were sequenced per PCR reaction. For all genes, both maternal (>95% methylation at CpG-sites for *Snrpn*, *Peg3* and *Igf2r*, and 0% methylation for *H19*) and paternal alleles (0% methylation for *Snrpn*, *Peg3* and *Igf2r*, and 100% methylation for *H19*) could be amplified in cumulus cells (results not shown).

DNA methylation patterns at imprinted genes in oocytes

The bisulphite sequencing technique leads to an important loss of DNA (>85%) and subsequently, to the amplification of only a limited number of different alleles when performed on small numbers of cells (Grunau *et al.*, 2001). Therefore, sequence polymorphisms (outside CpG-sites) between clones with similar methylation patterns were examined to ensure that only different alleles were included in the results for the oocytes. In total, approximately 672 clones were examined (192 for the superovulated MII oocytes; 448 for the MII oocytes after follicle culture and 32 for the oocytes from early pre-antral follicles), but only clones representing different alleles are shown in the results. Three up to eight different alleles could be obtained per gene for each oocyte pool.

Metaphase II oocytes from superovulated adult animals. The DNA methylation pattern of the DMRs of *Snrpn, Peg3, Igf2r* and *H19* has been described previously for superovulated metaphase II oocytes in adult female mice (Lucifero *et al.,* 2002). Results of the DNA methylation analysis of DMRs of *Snrpn, Peg3, Igf2r* and *H19* in our study are presented in Figs. 1-4 (A). All clones showed the previously described DNA methylation pattern. As expected, *H19* was found to be unmethylated in MII oocytes and this also served as a control to exclude the presence of somatic cell contamination (paternal alleles display a fully methylated pattern at CpG-sites).

For *Snrpn, Peg3* and *Igf2r,* the percentage of methylation at CpG-sites was close to 100% as expected: 99.6%, 98.7% and 100% respectively.

Metaphase II oocytes obtained after *in vitro* follicle culture and *in vitro* maturation in the presence of 10 IU/L r-FSH. Results of DNA methylation analysis of DMRs of *Snrpn*, *Peg3*, *Igf2r* and *H19* are presented in Figs. 1-4 (B). *H19* was unmethylated in MII oocytes (0.7% methylation at potential methylation sites). For *Snrpn*, *Peg3* and *Igf2r*, the percentage of methylation at CpG-sites was close to 100% as in the *in vivo* grown oocytes: 98.6%, 99.4% and 97.1 % respectively.

Metaphase II oocytes obtained after *in vitro* follicle culture and *in vitro* maturation in the presence of 100 IU/L r-FSH. Results of DNA methylation analysis of DMRs of *Snrpn*, *Igf2r* and *H19* are presented in Figs. 1,3 and 4 (C). *H19* was unmethylated in MII oocytes (2.2% methylation at potential methylation sites). For *Snrpn* and *Igf2r*, the percentage of methylation at CpG-sites was 99.7% and 98.6% respectively.

Statistical analysis of DNA methylation percentages at imprinted genes in MII oocytes. For *Snrpn, Igf2r and H19* no significant difference in DNA methylation percentage was noted between superovulated MII oocytes and MII oocytes after *in vitro* follicle culture in the presence of 10 IU/L r-FSH or in the presence of 100 IU/L r-FSH, nor between MII oocytes from both culture conditions (10 and 100 IU/L r-FSH).

Likewhise, no statistically different DNA methylation percentage was noted for *Peg3* between superovulated MII oocytes and MII oocytes after follicle culture in the presence of 10 IU/L r-FSH.

Oocytes from early pre-antral follicles in 13-14 day old mice. *H19* was unmethylated (0% methylation at potential methylation sites), excluding the presence of somatic cell contamination (results not shown). *Snrpn* showed 50.8% methylation at potential methylation sites (Fig. 1D).

Oocyte diameter, MII rate and developmental capacity of MII oocytes after in vitro follicle culture and in vitro maturation in the presence of 10 and 100 IU/L r-FSH

The mean diameter of fully grown germinal vesicle-stage oocytes was significantly larger for *in vivo* grown oocytes than for oocytes derived from *in vitro* follicle culture (at day 12) in the presence of 10 IU/L or 100 IU/L r-FSH: mean (SD) oocyte

TABLE 1

OOCYTE DIAMETER, MII RATE AND DEVELOPMENTAL CAPACITY OF OOCYTES

oocyte source	n follicles	mean (SD) oocyte diameter (μM)[1]	mean (SD) MII rate (%)	n oocytes[2] fertilized	2-cell/oocyte (%)	blastocyst/2-cell (%)	n transferred blastocysts	newborn/transfer (%)
In vitro follicle culture 10 IU/L r-FSH	672	71.6 (2.5)	92 (6.1)	550	52.0	54.3	208	3.4
In vitro follicle culture 100 IU/L r-FSH	658	71.6 (2.5)	94 (7.9)	555	53.5	46.8	-	-
In vivo grown	56	84.8 (4.6)	-	1013	89.0	48.5	199	10.0

Obtained from *in vitro* follicle culture and IVM in the presence of 10 or 100 IU/L r-FSH and of *in vivo* grown oocytes

[1] oocytes from antral follicles at day 12 (*in vitro* follicle culture) or from antral follicles of 25 day-old mice (*in vivo* grown)

[2] oocytes for fertilization experiments were obtained from other follicle cultures than those presented in the table for oocyte diameter and MII rate
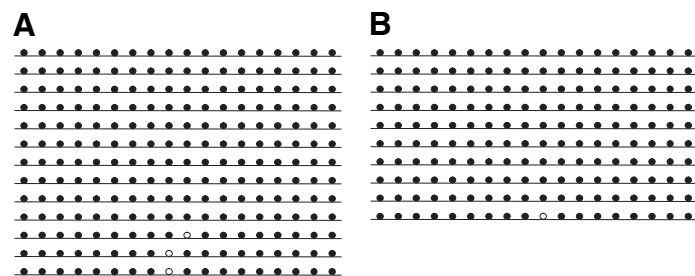
diameter was respectively 84.8 (4.6) µM, 71.6 (2.5) µM and 71.6 (2.5) µM; p<0.0001 for both comparisons (see Table 1). Mean oocyte diameters at day 12 were not significantly different for *in vitro* follicle culture and IVM between both r-FSH conditions.

Similarly, the mean MII rate was not significantly different between both r-FSH conditions: mean (SD) MII rate was 92 (6.1) % in the presence of 10 IU/L r-FSH and 94 (7.9) % in the presence of 100 IU/L r-FSH.

Results on the developmental capacity of *in vivo* grown (and superovulated) MII oocytes and MII oocytes after *in vitro* follicle culture and IVM are presented in Table 1. These data are not based on the follicle cultures used for the present study, but are laboratory data derived from multiple follicle cultures performed over the last 2 years. After fertilization, the 2-cell stage/oocyte rate was lower for MII oocytes obtained from *in vitro* follicle culture in the presence of 10 or 100 IU/L r-FSH than for *in vivo* grown and superovulated MII oocytes: respectively 52%, 53.5% and 89% (p-values<0.0001), but the blastocyst/2-cell rate was similar in the 3 groups: respectively 54.3%, 46.8% and 48.5% (p>0.05). The newborn/blastocyst transfer rate was lower for MII oocytes after follicle culture in the presence of 10 IU/L r-FSH than for *in vivo* grown and superovulated MII oocytes: respectively 3.4% and 10.0%, p=0.01. No blastocyst transfer experiments were performed for MII oocytes derived from *in vitro* follicle culture in the presence of 100 IU/L r-FSH (the policy was to consider only physiological r-FSH doses for transfer experiments).

Discussion

In this study, we compared DNA methylation at DMRs of 4 imprinted genes in mouse MII oocytes after prolonged *in vitro* follicle culture and *in vitro* oocyte maturation with *in vivo* grown oocytes after PMSG/hCG superovulation. The mouse follicle culture system allows oocytes to be grown from early pre-antral follicles up to fertilisable MII oocytes in a reproducible way during a 13-day culture period (Cortvrindt and Smitz, 2002). Our results indicate that oocytes derived from this follicle culture system have a lower diameter than *in vivo* grown oocytes as described previously for other follicle culture systems (Eppig and O'Brien, 1998). MII rates are above 90% and healthy offspring can be obtained from these oocytes, although at a lower rate than from *in vivo* grown oocytes.
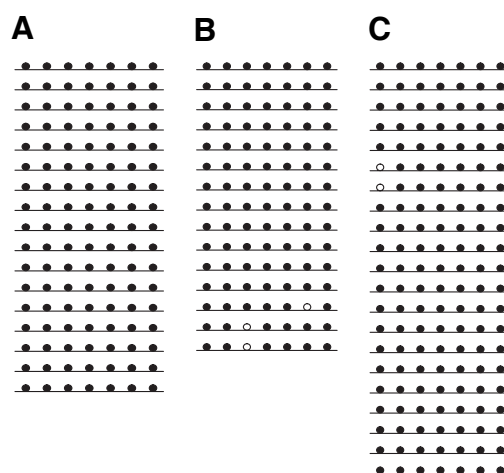
We found that DNA methylation at CpG-sites of DMRs of *Snrpn*, *Peg3* and *Igf2r* in *in vivo* grown, PMSG/hCG superovulated oocytes was close to 100%, as described previously for a different mouse strain (Lucifero *et al.*, 2002). The methylation pattern in MII oocytes after prolonged *in vitro* follicle culture and oocyte maturation in the presence of 10 IU/L r-FSH was comparable to the superovulated MII oocytes. Previous studies have shown that in early pre-antral follicles, DNA methylation at DMRs of *Snrpn*, *Peg3* and *Igf2r* is not fully established (Lucifero *et al.*, 2004; Hiura *et al.*, 2006). These findings were confirmed in our study for *Snrpn* in oocytes from early pre-antral follicles. Therefore, the correct DNA methylation pattern of *Snrpn*, *Peg3* and *Igf2r* in our study suggests that imprints for these 3 genes are correctly established under the actual *in vitro* follicle culture conditions and maintained in MII oocytes harvested at 18h post hCG.

A recent study in the same mouse strain suggested that prolonged IVM of oocytes in mouse (28 h of *in vitro* culture) leads to a loss of DNA methylation at the imprinted gene *Peg1*, suggesting that prolonged culture may result in a loss of previously acquired DNA methylation under certain culture conditions (Imamura *et al.*, 2005). However, 28h of *in vitro* maturation is excessively long, considering the fact that at 16h already, a maximum proportion of oocytes reach maturation, and that at 21h post hCG aging effects are visible on the spindle apparatus (Segers *et al.*, 2008).

The DNA methylation pattern at CpG-sites for *H19* was 0% in the *in vivo* grown superovulated oocytes as described previously (Lucifero *et al.*, 2002). Similarly, 0.7% methylation was found for *H19* in MII oocytes obtained with the follicle culture system in the presence of 10 IU/L r-FSH. The imprinted gene *H19* is not methylated during oogenesis, but was used in this study for two reasons: first, a non-methylated pattern at CpG-sites excluded the presence of cumulus cell contamination in the analyzed oocyte pools; second, hypermethylation at *H19* has been described in oocytes after IVM in human (Borghol *et al.*, 2006) and

Fig. 2 (Left). DNA methylation of *Peg3* (promoter and exon 1 region), (A) *in* in vivo *grown and PMSG/hCG superovulated MII oocytes, and* **(B)** *in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 10 IU/L r-FSH. For details, see Fig. 1 and its footnote.*

Fig. 3 (Right). DNA methylation of *Igf2r* (DMR2), (A) *in* in vivo *grown and PMSG/hCG superovulated MII oocytes;* **(B)** *in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 10 IU/L r-FSH and* **(C)** *in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 100 IU/L r-FSH. For details, see Fig. 1 and its footnote.*

after gonadotrophin superovulation with high doses in human and mouse (Sato *et al.*, 2007), suggesting that *H19* is susceptible to aberrant methylation in oocytes after *in vitro* culture or superovulation. The *H19* CTCF1-2 region was recently shown to be particularly susceptible to aberrant methylation after IVF and embryo culture in mouse (Fauque *et al.*, 2007). However, this *H19* DMR did not show aberrant methylation after *in vitro* follicle culture and *in vitro* oocyte maturation in our study.

Our results are in contrast with a previous study, performed in the same mouse strain, suggesting that a 12-day *in vitro* follicle culture can lead to a loss of methylation at *Igf2r* DMR and a gain of methylation at *H19* DMR in fully grown germinal vesicle-stage oocytes (Kerjean *et al.*, 2003). In the latter study, the culture medium used was α-minimal essential medium as in our study, but r-FSH was added in a concentration (100 IU/L) ten times higher than in this study (10 IU/L). Previous studies have established that the minimal effective dose of r-FSH under the accompanying culture parameters is around 5 IU/L. The dose of 10 IU/L of r-FSH could be considered physiological, while 100 IU/L exceeds the minimal needs by a factor of twenty (Adriaens *et al.*, 2004). Our present results confirm that *in vitro* follicle culture in the presence of 100 IU/L r-FSH does not result in larger oocyte diameters or in higher MII rates, nor in a higher blastocyst rate, when compared to the physiological r-FSH dose of 10 IU/L. Studies on the effects of superovulation on DNA methylation have shown conflicting results. Superovulation with several doses of PMSG has been linked to aberrant DNA methylation of *H19* in mouse oocytes (Sato *et al.*, 2007). Two recent studies in mouse have shown that superovulation may induce aberrant expression of imprinted genes in blastocysts (*H19*) or 9.5 dpc placentae (*H19* and *Snrpn*), (Fauque *et al.*, 2007; Fortier *et al.*, 2008). However, DNA methylation patterns at the studied imprinted genes were not altered, suggesting that superovulation interferes with the maintenance of imprinting after fertilization through mechanisms distinct from altered DNA methylation at the studied regulatory sequences. Therefore, we studied the effect of high doses of r-FSH (100 IU/L) in our follicle culture system on DNA methylation at regulatory sequences of *Snrpn*, *Igf2r* and *H19* in MII oocytes. We found no alterations in DNA methylation levels, demonstrating that high doses of FSH do not induce aberrant DNA methylation at the studied regulatory sequences in oocytes.

Another difference between the present study and the study from Kerjean is that follicle culture was performed in small culture droplets under mineral oil in the latter (Kerjean *et al.*, 2003). Perhaps prolonged culture in small medium droplets under mineral oil leads to an accumulation of toxic metabolic components such as ammonium in the culture medium. Ammonium has been linked with aberrant imprinting in *in vitro* pre-implantation embryo culture (Gardner and Lane, 2005). Follicles in their exponential growth phase have several thousands of metabolically active cells (Cortvrindt and Smitz, 1998) and might perhaps generate more or less toxic end products, depending on the hormonal composition of medium (FSH, insulin) and the oxygen exposure (Eppig *et al.*, 2000).

There is growing concern that ART may lead to an increased incidence of rare imprinting disorders in children. Furthermore, methylation analysis of these affected ART-children points to a hypomethylation of the maternal allele at certain imprinted loci (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003). Therefore, there is need for identifying factors possibly involved in aberrant imprinting in oocytes. We hypothesize that the mouse follicle culture model is a sensitive system that can be used in future experiments to study the influence of critical elements in culture conditions on imprinting establishment. The follicle culture bioassay might determine the critical stages of oocyte growth and maturation during which aberrant imprinting may be induced, by exposing follicles at different days of the culture, in a more reproducible and precise way than could be done by *in vivo* exposure. Ultimately, the model could be used for the optimalization of prolonged culture conditions of oocytes for clinical IVM.

In conclusion, MII oocytes grown in a well-characterized long-term pre-antral follicle culture system, do show a normal DNA methylation at regulatory sequences of key imprinted genes in the presence of physiological and high dose r-FSH. The bisulphite sequencing technique is associated with a substantial loss of DNA, commonly resulting in amplification of only a few alleles. Therefore, it was decided to perform a high number of independent experiments and conclusions were based on a high number of different alleles.

To the best of our knowledge, this is the first study that shows that prolonged follicle culture and IVM can generate MII oocytes with normal DNA methylation
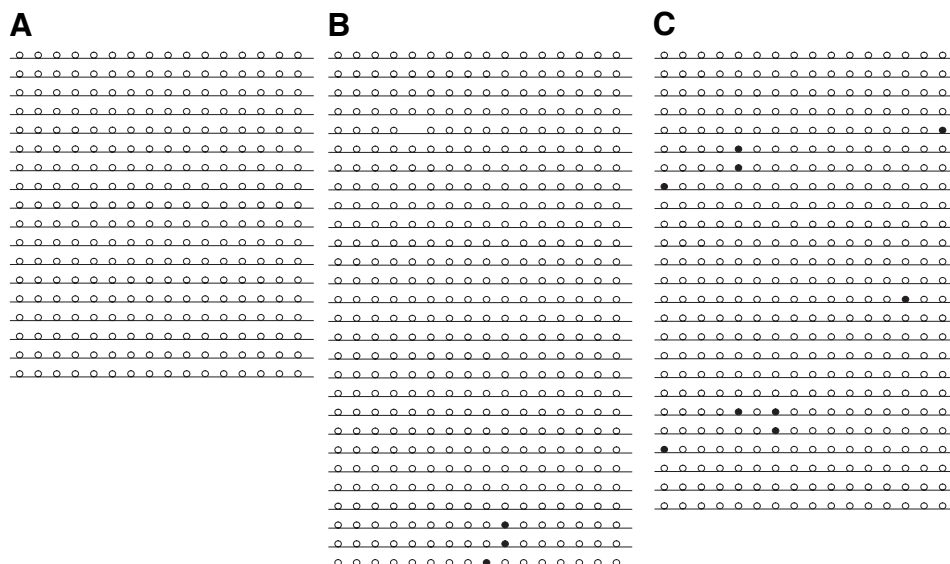
Fig. 4. DNA methylation of *H19* (DMR containing the CTCF 1-2 region). *(A) in* in vivo *grown and PMSG/hCG superovulated MII oocytes; (B) in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 10 IU/L r-FSH and (C) in MII oocytes after prolonged follicle culture and* in vitro *oocyte maturation in the presence of 100 IU/L r-FSH. For details: see Fig. 1 and its footnote.*

patterns at regulatory sequences of key imprinted genes even in the presence of high doses of FSH.

We speculate that deviations from normal concentrations of key components of culture medium and/or deviations from the biological time scales of critical growth and maturation processes might compromise normal establishment and/or maintenance of imprinting. Considering the low frequency of imprinting disorders after ART and the potential etiologies, it is extremely difficult to explore cause-effect relationships from clinical data. Precisely defined *in vitro* models are powerful tools for defining the edges of therapeutic interventionism.

Materials and Methods

Oocyte and cumulus cell collection

This study was performed with F1 mice (C57BL/6J x CBA/Ca; Harlan, The Netherlands), housed and bred according to the national standards for animal care, and approved by the Ethical Committee for animal experiments of the Vrije Universiteit Brussel (Project Nr. 01-395-1).

Adult 8-week old female mice were superovulated by intraperitoneal injection of 5 IU of pregnant mares' serum gonadotrophin (PMSG, Intervet, Mechelen, Belgium), followed 48h later by intraperitoneal injection of 5 IU human chorionic gonadotrophin (hCG, Intervet). Oocytes-cumulus cell complexes containing MII oocytes were removed from the oviducts 14h after hCG injection and collected into L15 Leibovitz-glutamax medium supplemented with 10% Heat Inactivated Foetal Bovine Serum (HIA FBS), 100 µg/ml of streptomycin, and 100 IU/ml of penicillin (Invitrogen, Merelbeke, Belgium). Cumulus cells were removed from the superovulated MII oocytes with 1 mg/mL hyaluronidase (Roche Diagnostics, Brussels, Belgium). The MII oocytes used in the experiments were obtained from 9 superovulated mice. Per superovulated female, 30 to 45 MII oocytes were obtained.

Metaphase II oocytes were obtained after *in vitro* follicle culture as described previously (Cortvrindt and Smitz, 2002). Briefly, early pre-antral follicles with a diameter between 100 and 130 µm were mechanically isolated from the ovaries of 13-14-day-old F1 mice in L15 medium (Fig. 5). The follicle culture medium consisted of α-minimal essential medium (α-MEM, Invitrogen) supplemented with 5% HIA FBS, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS, Sigma, Bornem, Belgium) and 10 IU/L or 100 IU/L recombinant follicle-stimulating hormone (r-FSH, Gonal-F®, Serono, Benelux). Follicles were individually cultured in 75 µl medium in 96-wells (Costar, Belgium) for a 12-day period to grow to antral follicles. Follicles were cultured in an incubator at 37°C, 100% humidity and 5% CO_2 in air. Exchanging part of the medium (30 µl) was done on days 4, 8 and 12. At the end of the 12-day culture period, an ovulatory stimulus with 1.2 IU/ml recombinant human chorionic gonadotrophin (r-hCG, Ovitrelle, Serono,

Benelux) supplemented with 4 ng/ml recombinant epidermal growth factor (r-EGF, Roche Diagnostics) was given. Oocyte-cumulus cell complexes containing MII oocytes were collected into L15 medium 18 hours after r-hCG/r-EGF. The MII oocytes used for the experiments were obtained from 8 independent repeat cultures (4 for the 10 IU/L r-FSH and 4 for the 100 IU/L r-FSH condition) involving 4 mice per culture experiment.

Oocytes were washed free from somatic cells by transfer through three washes of L15 medium with a mouth-controlled glass pipette. One hundred to 150 MII oocytes per culture were pooled. One hundred oocytes from early pre-antral follicles (with a diameter between 100 and 130 µm) in ovaries of four 13-14-day-old F1 mice were collected as described previously (Lucifero *et al.*, 2002) and pooled.

Special care was taken that no cumulus cells should contaminate the oocyte samples. Furthermore, *H19* methylation analysis was performed to ensure the absence of cumulus cell contamination (fully methylated *H19* strands suggest cumulus cell contamination) and none of the examined clones showed a methylated pattern.

Cumulus cells were collected separately (cumulus cells from 1 COC complex per sample) to serve as somatic cell controls for validation of the bisulphite sequencing technique.
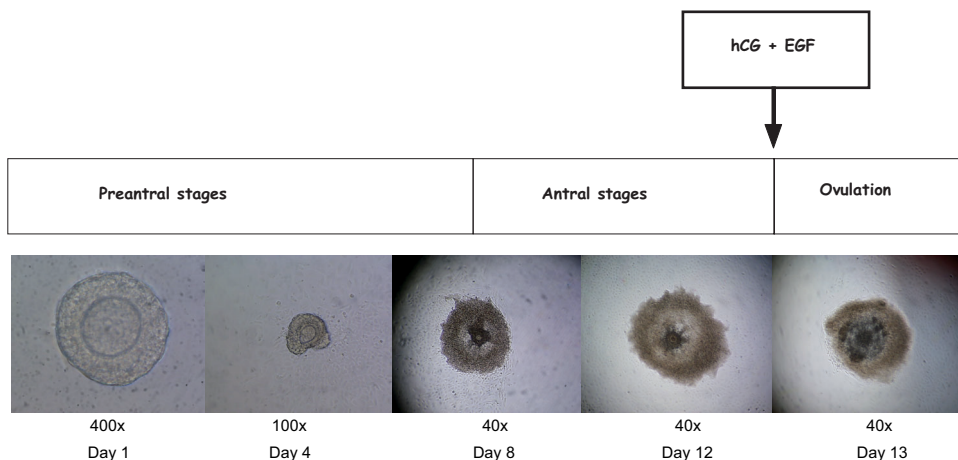
DNA methylation analysis and PCR

DNA was extracted from the oocyte pools (containing 100-150 oocytes per pool) using the QIAamp DNA Micro kit (Qiagen, Venlo, The Netherlands). Bisulphite treatment was performed with the EZ DNA Methylation kit (Zymogen Research, Orange, CA, USA). DNA was eluted in 10 µL of the kit's elution buffer, stored at -80°C and used within 2 weeks for PCR. Nested PCR was performed for amplification of DMRs of the imprinted genes *H19, Snrpn, Peg3* and *Igf2r*. For *H19, Snrpn* and *Igf2r*, PCR was performed on 3 (superovulation) or 8 (*in vitro* follicle culture conditions) independent samples of 100-150 MII oocytes with primers for bisulphite-treated DNA. For *Peg3*, PCR was performed on 3 (superovulation) or 4 (*in vitro* follicle culture in the presence of 10 IU/L r-FSH) independent samples of 100-150 MII oocytes. Furthermore, one PCR was performed for *Snrpn* and *H19* on the pool of 100 oocytes from early pre-antral follicles. Primer sequences for *Snrpn* DMR1, *Igf2r* DMR2, *Peg3* promoter and exon 1 region and *H19* DMR (containing the CTCF 1-2 region involved in imprinted expression of Igf2), have been previously described (Lucifero *et al.*, 2002). We analysed 16 cpG sites in a 422 bp fragment of *H19* (GenBank acc.nr. U19619, 1304-1726), 16 CpG sites in a 419 bp fragment of *Snrpn* (AF081460, 2151-2570), 18 CpG sites in a 286 bp fragment of *Peg3* (AF105262, 2770-3056) and 7 CpG sites in a 205 bp fragment of *Igf2r* (L06446, 796-1001), (Lucifero *et al.*, 2002).

For *H19, Snrpn* and *Peg3*, the first PCR reaction was performed in 50 µL and contained 2 µL bisulphite-modified DNA, 5 µL of 5x PCR buffer II, 5 µL of 2.5mM $MgCl_2$ (Applied Biosystems, Nieuwerkerk, The Nether-

Fig. 5. Representative picture of the *in vitro* growth of an early pre-antral follicle (type 3b) in defined conditions. *The thecal cells attach the pre-antral follicle to the bottom of the culture dish (day1 to 4), the granulosa cells break through the basal membrane (day 4) and proliferate. The granulosa cells differentiate into a follicular wall and a cumulus-corona part (day 8). A clear antral-like cavity has formed on day 12. The hCG / EGF stimulus on these follicles provokes the release of a mucified cumulus-corona complex (day 13) surrounding a metaphase II oocyte. Note that the oocyte diameter increases from 50-55 µm (diameter in type3b follicle) up to 72-75 µm (diameter before ovulation).*

		hCG + EGF

Preantral stages	Antral stages	Ovulation

400x	100x	40x	40x	40x
Day 1	Day 4	Day 8	Day 12	Day 13

lands), 0.25 µL of 2 mM dNTPs (Invitrogen), 0.5nµL of 60 µM for each primer solution and 0.4 µL AmpliTaq (Applied Biosystems). The second PCR reaction was performed in 25µL and contained 2 µL of first-round PCR product, 2.5 µL of 5x PCR buffer II, 2.5 µL of 2.5mM MgCl$_2$, 2.5 µL of 2 mM dNTPs, 0.5 µL of 60 µM primer solution and 0.2 µL AmpliTaq. First round PCR cycle conditions were 2 minutes at 94°C (pre-denaturation); 4 minutes at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C for 2 cycles; 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C for 35 cycles; 10 minutes at 72°C (final elongation). Second round PCR cycle conditions were similar, except for the first two cycles that were omitted.

For *Igf2r*, first and second round PCR was performed with the Advantage cDNA PCR Kit and Polymerase Mix (Westburg B.V., Leusden, The Netherlands) according to the instructions of the manufacturer. PCR cycle conditions were the same for first and second round PCR: 4.5 minutes at 95°C (pre-denaturation); 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C for 30 cycles; 5 minutes at 72°C (final elongation).

Cloning and sequencing

PCR products were separated on a 1% agarose gel; bands of interest were excised and purified with the Perfectprep Gel Cleanup kit (Eppendorf AG, Hamburg, Germany). DNA was subcloned using the TOPO TA Cloning kit (Invitrogen). Plasmid DNA was isolated with the QIAprep Miniprep kit (Qiagen). For each oocyte pool, sixteen clones were sequenced per gene using M13 Reverse and M13 Forward universal primers with the Big Dye Terminator kit and the ABI PRISM 3100 sequencer (Applied Biosystems). As a control for completeness of bisulphite modification, the conversion of cytosine residus outside CpGs was analysed in each sequence. Cytosines outside CpGs are unmethylated and should therefore be converted by bisulphite treatment. Only sequences with > 95% bisulphite conversion of cytosine residues outside CpGs were included in the results to exclude false results due to incomplete DNA modification by bisulphite.

Developmental capacity of MII oocytes after in vitro *follicle culture and* in vitro *maturation in the presence of 10 and 100 IU/L r-FSH*

In vivo grown and superovulated MII oocytes and MII oocytes derived from *in vitro* follicle culture were used for IVF and embryo culture as described previously (Adriaens *et al.*, 2004). Blastocysts were surgically transferred into the uterine horns of 8- to 12-week CD1, pseudopregnant females.

Statistics

For each imprinted gene, DNA methylation percentages of clones were compared in the several MII oocyte groups by Mann-Whitney U analysis. For *in vitro* follicle cultures, MII rate and oocyte diameters were compared between the 10 IU/L and 100 IU/L r-FSH conditions by Mann-Whitney U analysis. Oocyte diameters were also compared between *in vivo* grown oocytes and both *in vitro* follicle culture conditions by Mann-Whitney U analysis. Differences in developmental capacity (2-cell/oocyte rate, blastocyst/2-cell rate and newborn/blastocyst transfer rate) were examined between *in vivo* grown (and superovulated) oocytes and MII oocytes derived from *in vitro* follicle culture by the Chi-square test. P-values <0.05 were considered significant.

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