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 bisulphite 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

Abbreviations used in this paper: ART, assisted reproductive technology; DMR, differentially methylated region; FSH, follicle-stimulating hormone.
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 (Lucifer 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 (Bielsinska 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

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).

Likewise, 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 diameter (µm) 71.8 (2.5), 71.6 (2.5), 71.6 (2.5) and 71.6 (2.5) respectively for in vivo grown, 10 IU/L r-FSH, 100 IU/L r-FSH and in vitro follicle culture oocytes.

Table 1

<table>
<thead>
<tr>
<th>Oocyte source</th>
<th>n follicles</th>
<th>mean (SD) oocyte diameter (µm)</th>
<th>mean (SD) MII rate (%)</th>
<th>n oocytes fertilized</th>
<th>2-cell/oocyte (%)</th>
<th>blastocyst/2-cell (%)</th>
<th>n transferred blastocysts</th>
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<td>672</td>
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<td>52.0</td>
<td>54.3</td>
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<tr>
<td>In vitro follicle culture</td>
<td>658</td>
<td>71.6 (2.5)</td>
<td>94 (7.9)</td>
<td>555</td>
<td>53.5</td>
<td>46.8</td>
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<tr>
<td>In vivo grown</td>
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<td>84.8 (4.6)</td>
<td>-</td>
<td>1013</td>
<td>89.0</td>
<td>48.5</td>
<td>199</td>
<td>10.0</td>
</tr>
</tbody>
</table>

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 Table1. 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 *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 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 the studied regulatory sequences in oocytes. 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 mineral 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 at 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 (Epig 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 oocytes 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 optimization 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.
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 MI 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 MI oocytes with 1 mg/mL hyaluronidase (Roche Diagnostics, Brussels, Belgium). The MI 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-MEM 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 of 2.5mM MgCl2 (Applied Biosystems, Nieuwerkerk, The Netherlands) supplemented with 4 ng/ml recombinant epidermal growth factor (r-EGF, Roche Diagnostics) was given. Oocyte-cumulus cell complexes containing MI oocytes were collected into L15 medium 18 hours after r-hCG/r-EGF. The MI 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 MI 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 MI 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 MI 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 H19DMR (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 MgCl2 (Applied Biosystems, Nieuwerkerk, The Nether-
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/blastoctyst 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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