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# Culture of oocytes and risk of imprinting defects

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**BACKROUND:** Follicle culture and oocyte *in vitro* maturation (IVM) are emerging assisted reproductive technologies with potentially important future applications in the fertility clinic. There is concern that these technologies might interfere at the epigenetic level and, in particular, with genomic imprinting. The timely acquisition of correct imprinting patterns in oocytes and the maintenance of genomic imprinting after fertilization are both required for normal embryonic development.

**METHODS:** A systematic literature search in Pubmed was performed and all publications reporting on the effects of follicle culture, IVM or ovarian tissue culture on genomic imprinting were retained.

**RESULTS:** Mouse ovarian tissue culture studies, mouse *in vitro* follicle culture studies and a single bovine IVM study generally showed correct imprinted DNA methylation establishment in oocytes. Influences of treatment and suboptimal culture conditions in mouse follicle culture indicate that imprinting establishment in oocytes is a robust process. This is in contrast with preimplantation embryo culture-induced epigenetic defects reported in mice. For human IVM, no definitive conclusion on imprinting establishment can be drawn as well-designed studies are currently not available.

**CONCLUSIONS:** Animal models provide reassuring data on imprinting establishment in cultured oocytes, but further studies should assess the effect of oocyte culture on imprinting maintenance. Optimized IVM procedures should be assessed in well-designed human studies. Finally, epigenetic analysis should be performed in children born from pregnancies after IVM to draw definitive conclusions on the epigenetic safety of human IVM.

**Key words:** oocyte / culture / IVM / follicle culture / genomic imprinting

# Introduction

Follicle culture and oocyte *in vitro* maturation (IVM) are emerging assisted reproductive technologies (ART) with potentially important future applications for fertility preservation and reduction of hormone pretreatment in well-defined infertility patient groups (Smitz *et al.*, 2010, 2011).

Follicle culture technology for human clinical applications attracts much research attention as it represents an alternative to ovarian cortical tissue transplantation (Xu *et al.*, 2009). So far, the complete *in vitro* growth of follicles from the primordial or early pre-antral stages to maturity, and their subsequent fertilization and development into embryos which grow to term, has only been successful in the mouse model. In contrast, IVM or the use of *in vitro* culture systems for the maturation of collected immature oocytes prior to fertilization represents routine practice in cattle breeding programs. The technology was introduced for human infertility treatment in 1991 (Cha *et al.*, 1991), and since then over 1000 children have been born. Although the results of the children's follow-up are generally reassuring (Basatemur and Sutcliffe, 2011), it cannot be denied that *in vitro* oocyte development and maturation is a complex and challenging procedure and there is concern about the possible interference of these techniques with epigenetic mechanisms and, in particular, with genomic imprinting.

Genome imprinting is a phenomenon leading to a parent-of-origin-specific monoallelic expression of genes in diploid cells. For most genes, both the paternal and maternal alleles are actively transcribed. In contrast, a small number of so-called 'imprinted genes' are expressed from only the paternal or from only the maternal allele (Surani *et al.*, 1984; Reik and Walter, 2001). To date, around 100 imprinted genes have been identified in mouse and human, most of them residing in clusters sharing common *cis*-regulatory imprinting control regions (ICRs) (for a complete list of known imprinted genes, see hptt:/www.har.mrc.ac.uk/research/genomic_imprinting/ and http://igc.otago.ac.nz/home.html).

Imprinting explains why mammalian development requires both a paternal and maternal genome (McGrath and Solter, 1984; Surani *et al.*, 1984). A balanced (uniparental) expression of imprinted genes is indeed essential for normal embryo development, placental differentiation and pre- and post-natal growth, but also for normal neurobehavioural processes and metabolism (reviewed in Isles and Holland, 2005; Fowden *et al.*, 2006; Smith *et al.*, 2006). Furthermore, aberrant imprinting is linked to human imprinting syndromes such as Beckwith–Wiedemann (BWS), Prader–Willi (PWS) and Angelman (AS) syndromes and to cancer (reviewed by Lim and Maher, 2010; Uribe-Lewis *et al.*, 2011).

Imprinted genes are differently marked by epigenetic modifications in the parental alleles so that only one of the parental alleles is expressed. DNA methylation is the best characterized epigenetic modification that controls genomic imprinting (reviewed by Reik and Walter, 2001; Li, 2002). The ICRs of imprinted genes are usually associated with tandem repeat DNA sequence structures and with differentially methylated regions (DMRs). The specific regulation of imprinted gene expression has been reviewed in detail elsewhere (Ideraabdullah *et al.*, 2008).

Imprints are erased in primordial germ cells when the bulk of DNA demethylation occurs (Szabo and Mann, 1995; Kato *et al.*, 1999; Hajkova *et al.*, 2002; Lee *et al.*, 2002; Szabo *et al.*, 2002) allowing imprints to be subsequently reset during gametogenesis in a sex-specific manner (Fig. 1). For most known imprinted genes, DNA methylation at ICRs is acquired at the maternal allele during oogenesis. At only four of the known ICRs in the mouse (*H19*, *Rasgrf1*, *Dlk1/Dio3*, *Zdbf2*), DNA methylation is acquired during spermatogenesis (Davis *et al.*, 1999; Li *et al.*, 2004; Kobayashi *et al.*, 2009). The time of imprinting acquisition is different between the male and the female germline in mouse. In the male, DNA methylation at DMRs starts prenatally in prospermatogonia and is completed post-natally at the pachytene stage of meiosis (Li *et al.*, 2004). In contrast, imprinted DNA methylation occurs only after the pachytene stage of meiosis I in the post-natal growing mouse oocyte.

During post-natal mouse oogenesis, imprinting establishment occurs asynchronously at different imprinted genes (Fig. 2), while oocytes are arrested at prophase I during the transition from primordial to antral follicle stages (Obata and Kono, 2002; Lucifero *et al.*, 2004; Hiura *et al.*, 2006), and this acquisition of DNA methylation correlates with an increase in oocyte diameter (Hiura *et al.*, 2006). Bovine oocytes also acquire DNA methylation imprints during the post-natal growth period in an oocyte size-dependent manner (Fig. 3, O'Doherty *et al.*, 2012).

DNA methylation at ICRs in the female germline requires *de novo* DNA methyltransferase Dnmt3a and a cofactor without intrinsic catalytic activity, Dnmt3L (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Kaneda *et al.*, 2004). The mechanism that targets the methylation complex to ICRs is unclear but permissive histone modifications seem to be necessary and a transcription-based mechanism in the female germline has been identified for the mouse Gnas locus (Ooi *et al.*, 2007; Chotalia *et al.*, 2009; Ciccone *et al.*, 2009).

To allow full-term development, the differential DNA methylation patterns of imprinted genes should not only be accurately established during gametogenesis, but also subsequently correctly maintained despite genome-wide changes in DNA methylation during preimplantation. Several oocyte-expressed genes, such as Dnmt1o, Zfp57, Stella and Mbd3 have been shown to be required for this imprinting maintenance during preimplantation development (Howell *et al.*, 2001; Nakamura *et al.*, 2007; Reese *et al.*, 2007; Li *et al.*, 2008). Thereafter, imprints are faithfully propagated in somatic cells during cell divisions.

From what is described above, it may be anticipated that the manipulation of gametes or embryos during ART might interfere with the establishment and/or maintenance of imprinting. Indeed, *in vitro* preimplantation embryo culture has been linked to a failure of imprinting maintenance in animal models (Doherty *et al.*, 2000; Khosla *et al.*, 2001; Young *et al.*, 2001; Mann *et al.*, 2004; Rivera *et al.*, 2008; Suzuki *et al.*, 2009; Market-Velker *et al.*, 2010a). Data in human are inconsistent: several studies have suggested a possible link between ART and rare imprinting syndromes (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Ørstavik *et al.*, 2003; Halliday *et al.*, 2004; Chang *et al.*, 2005; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Sutcliffe *et al.*, 2006; Bowdin *et al.*, 2007; Gomes *et al.*, 2007; Lim *et al.*, 2009), although other studies have not found an association (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). Strikingly, however, molecular analysis revealed that nearly all of these BWS cases were associated with a loss of DNA methylation on the maternal allele at the ICR regulating the growth-related *KCNQ1* domain (KvDMR1),

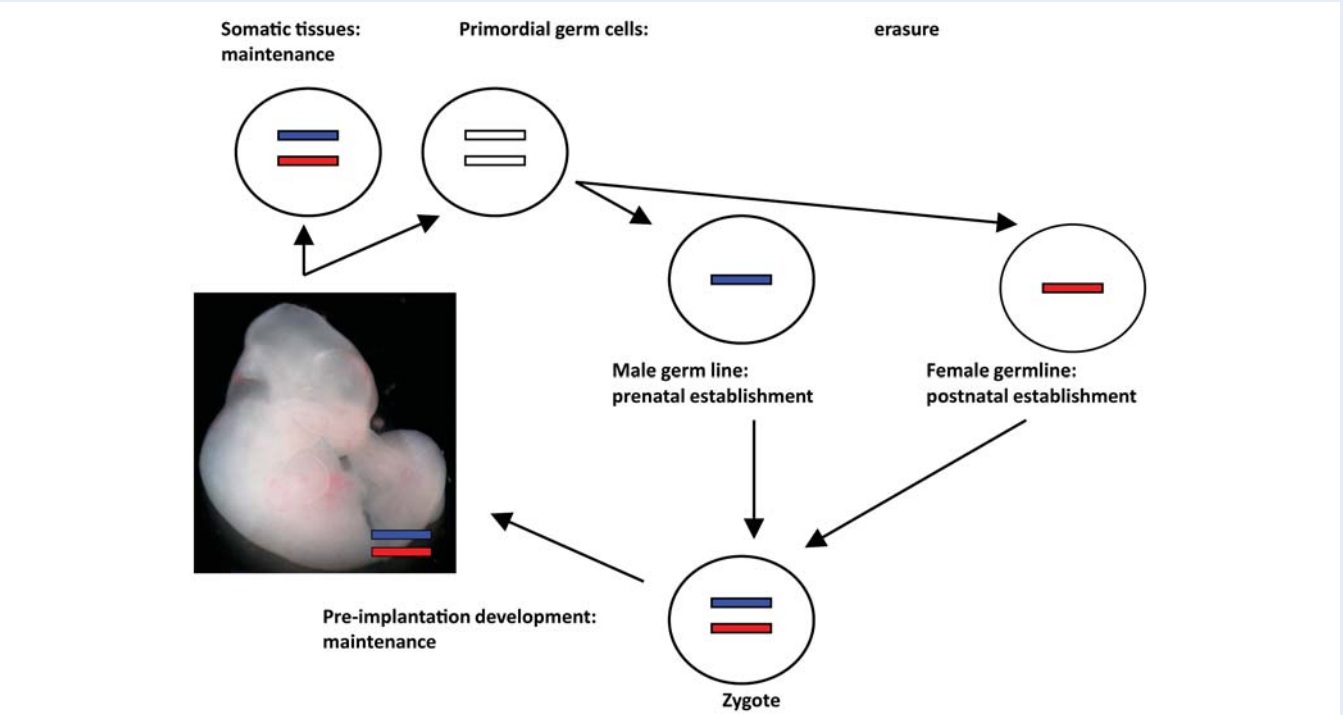


**Figure 1** Schematic representation of erasure, establishment and maintenance of genomic imprints in mammalian development: DNA methylation at ICRs is erased in primordial germ cells of the developing embryo at E11.5–12.5, and subsequently reset in a sex-specific manner during gametogenesis. Imprinted DNA methylation is maintained in somatic cells post-fertilization despite genome-wide DNA methylation changes during preimplantation development; red bars: maternal imprints; blue bars: paternal imprints.

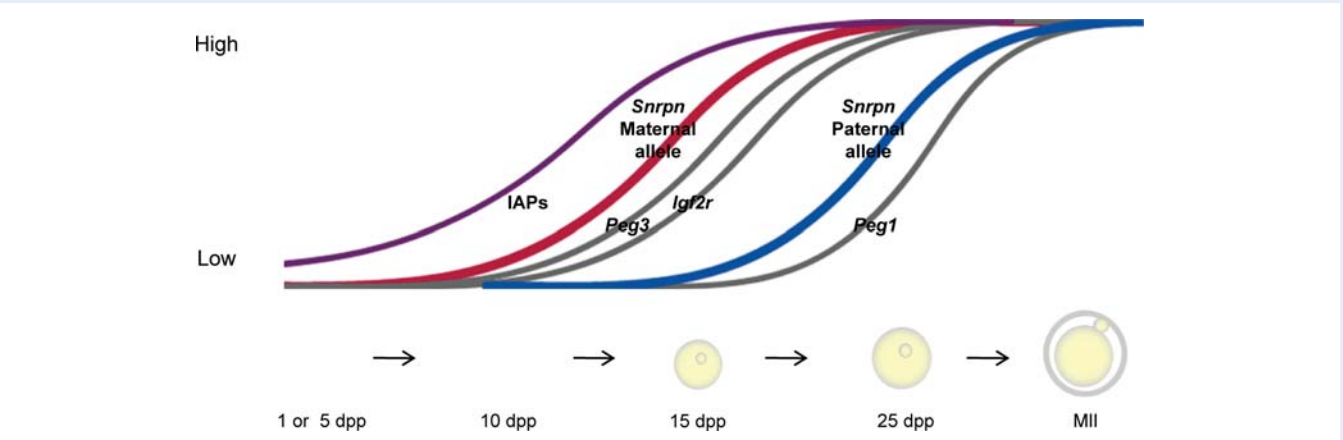


**Figure 2** Asynchronous methylation acquisition of maternally methylated imprinted genes: imprinting is established at a specific time during oocyte growth from the primary to antral follicle stage for *Peg3*, *Igf2* and *Mest* (also known as *Peg1*). For *Snrpn*, the maternally inherited allele acquires methylation before the paternal allele, indicating that another epigenetic mark (in the absence of DNA methylation) may be retained at the DMR that still allows the parental origin of alleles to be distinguished. Methylation acquisition at the non-imprinted intracisternal A-particles (IAPs) is also depicted. dpp = days post-partum. Reproduced with permission from Lucifero *et al.* (2004).

whereas this epigenetic abnormality is found in only around 50% of sporadic BWS patients (Lee *et al.*, 1999; Engel *et al.*, 2000).

Consequently, there is concern that *in vitro* culture and maturation might interfere with the acquisition of genome imprinting during oogenesis and/or might affect the competence of the oocyte for imprinting maintenance after fertilization.

The aim of this article is to review the current knowledge on the possible effects of *in vitro* culture of oocytes on genomic imprinting.

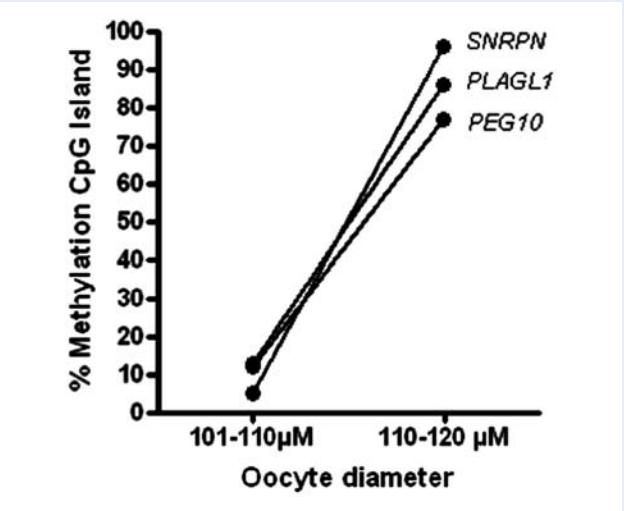


**Figure 3** Methylation acquisition of maternally imprinted genes in bovine oocytes occurring in an oocyte size-dependent manner: the percentage of CpG Island methylation is depicted in oocytes measuring 101–110 μM (pre-antral stage) and in oocytes measuring 110–120 μM for *SNRPN*, *PEG10* and *PLAGL1* (O'Doherty *et al.*, 2012).

# Methods

## Search criteria

A systematic literature search in Pubmed was performed using the keywords 'follicle culture', '*in vitro* maturation', 'IVM', 'oocyte culture', 'oocyte *in vitro*' and 'imprinting'; and 'DNA methylation' in various combinations with no limits applied. All publications reporting on the effects of follicle culture, IVM or ovarian tissue culture on genome imprinting were retained.

## The bisulphite sequencing method for DNA methylation analysis

Some caution is necessary when interpreting DNA methylation analysis at ICRs of imprinted genes in oocytes. The bisulphite sequencing technique is the widely used 'gold standard' technique to study DNA methylation patterns at DMRs of imprinted genes. The principle of the technique is based on the different sensitivity of cytosine (C) and 5-methylcytosine (5mC) to deamination by bisulphite: C undergoes a deamination to uracil (U); in contrast, 5mC does not react with bisulphite. DNA is subsequently amplified by PCR with primers specific for bisulphite-converted DNA. During PCR all uracils (U), which are bisulphite converted Cs are amplified as thymine (T) and 5mC is amplified as C.

To determine DNA methylation patterns at a single base pair and at single molecule resolution, the PCR product is subcloned in a vector and individual clones are sequenced separately. As an alternative to subcloning and sequencing, pyrosequencing allows a reliable determination of the percentage of methylation at a limited number of CpGs from the ratio of T and C at each CpG position.

The bisulphite sequencing technique may be prone to PCR and/or cloning bias (Warnecke *et al.*, 2002). In most cases, the PCR bias is towards a preferential amplification of unmethylated DNA strands because methylated DNA has a higher CpG content after bisulphite conversion, which raises the melting temperature and which may increase secondary structure formation possibly resulting in a lower PCR efficiency (Warnecke *et al.*, 1997). A preferential cloning of either methylated or unmethylated strands has also been described (Warnecke *et al.*, 2002). Therefore, the DNA methylation analysis should be validated, e.g. on somatic cells (containing 50% methylated and 50% unmethylated DNA templates) to exclude a bias towards unmethylated or methylated DNA strands.

Moreover, the technique is associated with a substantial loss of DNA, commonly resulting in amplification of only a few alleles when performed on DNA from pools with limited cell numbers and necessitating sufficient repeat experiments to detect rare events (Grunau *et al.*, 2001).

To overcome the latter two limitations, the bisulphite sequencing technique has been successfully applied to single human oocytes imbedded in agarose beads (Geuns *et al.*, 2003) and an elegant limiting dilution (LD) technique has been developed (El Hajj *et al.*, 2011).

However, the most critical issue when applying the bisulphite sequencing technique to oocytes is the avoidance of somatic cell contamination. Extreme care should be taken to completely remove cumulus cells from oocytes as these will inevitably result in a bias (with paternal and maternal alleles from somatic cells showing an opposite methylation pattern and being wrongfully interpreted as aberrant or normal oocyte imprinting patterns, respectively). Somatic cell contamination events were found to underlie irreproducible results. Somatic cell or environmental contamination occurring in some reported studies might therefore be an explanation for at least some of the discrepant findings of culture- or ovulation induction-induced effects on imprinting establishment in oocytes.

# Results

A summary of literature data on imprinting establishment in *in vitro* cultured oocytes is presented in Table I.

## Animal models for *in vitro* follicle culture and oocyte imprint establishment

### *Mouse* in vitro *follicle culture*

A mouse *in vitro* follicle culture system (Fig. 4; Cortvrindt and Smitz, 2002) was employed to study the influence of *in vitro* follicle culture on imprinting establishment at the maternally methylated genes *Snrpn*, *Igf2r*, *Mest* (also known as *Peg1*), *Peg3* and the paternally methylated *H19* in oocytes. These genes were chosen because of their importance in normal development and post-natal behaviour (Lau *et al.*, 1994; Lefebvre *et al.*, 1998; Li *et al.*, 1999; Liu *et al.*, 2008; Gabory *et al.*, 2009). Furthermore, aberrant imprinting of these genes after ART had been reported in animal models (Doherty *et al.*, 2000; Khosla *et al.*, 2001; Young *et al.*, 2001; Mann *et al.*, 2004; Rivera *et al.*, 2008; Suzuki *et al.*, 2009). The follicle culture system allows the growth of oocytes from isolated early pre-antral follicles from 12 dpp old C57BL/6J x CBA/Ca mice up to fertilization-competent metaphase II (MII) oocytes in a reproducible way during a 13-day culture period. Studies in different mouse strains have shown that in oocytes at the early pre-antral follicle stage, as used for the follicle culture system, DNA methylation at the DMRs of *Snrpn*, *Peg3*, *Mest* and *Igf2r* is not fully established (Lucifero *et al.*, 2004; Hiura *et al.*, 2006; Anckaert *et al.*, 2009a). The methylation patterns of these four genes and of the paternally methylated H19 gene, as assessed by bisulphite sequencing in pools of MII oocytes from *in vitro* follicle culture were comparable with those of *in vivo* grown superovulated MII oocytes (Anckaert *et al.*, 2009a) and showed the methylation patterns as previously described in *in vivo* grown mouse oocytes (Fig. 2;

**Table I Overview of literature data on imprinting establishment in *in vitro* cultured oocytes.**

| Reference | Species/ strain | Age | Culture medium | Culture conditions | Gene | DNA methylation |
|---|---|---|---|---|---|---|
| Shen *et al.* (2007) | Mouse, CD-1 | Prepub | α-MEM with 5% FCS | *In vitro* follicle culture (pre-antral)<br>100 IU/L FSH<br>20 µl medium droplet under mineral oil | *Igf2r* | = |
| Kerjean *et al.* (2003) | Mouse, C57BL/ 6JxCBA | Prepub | α-MEM with 5% FCS | *In vitro* follicle culture (pre-antral)<br>100 IU/L FSH<br>20 µl medium droplet under mineral oil | *Igf2r*<br>*Mest*<br>*H19* | ↓<br>↓<br>↑ |
| Anckaert *et al.* (2009a) | Mouse, C57BL/ 6JxCBA | Prepub | α-MEM with 5% FCS | *In vitro* follicle culture (pre-antral)<br>75 µl medium without mineral oil<br>10 IU/L r-FSH | *Igf2r*<br>*Snrpn*<br>*H19*<br>*Peg3* | =<br>=<br>=<br>= |
| | | | | 100 IU/L r-FSH | *Igf2r*<br>*Snrpn*<br>*H19* | =<br>=<br>= |
| Anckaert *et al.* (2009b) | Mouse, C57BL/ 6JxCBA | Prepub | α-MEM with 5% FCS | *In vitro* follicle culture (pre-antral)<br>10 IU/L r-FSH<br>Under mineral oil and ↑ NH4 | *Igf2r*<br>*Snrpn*<br>*H19* | =<br>=<br>= |
| Anckaert *et al.* (2010) | Mouse, C57BL/ 6JxCBA | Prepub | α-MEM with 5% FCS | *In vitro* follicle culture (pre-antral)<br>75 µl medium without mineral oil<br>10 IU/L r-FSH<br>Control culture medium (α-MEM) | *Mest* | = |
| | | | | Methyl donor restriction | *Igf2r*<br>*Snrpn*<br>*H19*<br>*Mest* | =<br>=<br>=<br>↓ |
| Trapphoff *et al.* (2011) | Mouse, C57BL/ 6JxCBA | Prepub | α-MEM with 5% FCS | *In vitro* follicle culture (pre-antral)<br>10 IU/L r-FSH<br>50 µl medium under mineral oil | *Igf2r*<br>*Snrpn*<br>*H19* | =<br>=<br>= |
| Barboni *et al.* (2011) | Ovine | Prepub | α-MEM with 2% FCS | *In vitro* follicle culture (pre-antral)<br>1 µg/ml ovine FSH<br>25 µl medium under mineral oil | *H19*<br>*IGF2R* | =<br>= |
| Heinzmann *et al.* (2011) | Bovine | Adult | TCM199, 1 mg/ml BSA | IVM<br>10 IU/ml eCG and 5 IU/ml hCG | *H19*<br>*PEG3*<br>*SNRPN* | =<br>=<br>= |
| | | | mSOF, 4 mg/ml BSA | IVM<br>10 IU/ml eCG and 5 IU/ml hCG | *H19*<br>*PEG3*<br>*SNRPN* | =<br>=<br>= |
| Colosimo *et al.* (2009) | Ovine | Adult | Not specified | IVM | *H19*<br>*IGF2R* | =<br>= |
| Imamura *et al.* (2005) | Mouse, C57BL/ 6JxCBA | Adult | M16 | *In vitro* aging of MII oocytes (recovered after *eCG/hCG*) for 28 h (up to 42 h post-hCG) | *Mest* | ↓ |
| Liang *et al.* (2008) | Mouse, Kunming | Adult | M16 | *In vitro* aging of MII oocytes (recovered after *eCG/hCG*) for 16 h (up to 29 h post hCG)<br>Denuded oocytes | *Mest*<br>*Snrpn* | =<br>↓ |
| | | | | Cumulus cell-enclosed oocytes | *Mest*<br>*Snrpn* | =<br>= |

*Continued*

**Table I** *Continued*

| Reference | Species/ strain | Age | Culture medium | Culture conditions | Gene | DNA methylation |
|---|---|---|---|---|---|---|
| Borghol *et al.* (2006) | Human | | Medicult supplemented with FSH, hCG and 10% patient serum | Rescue IVM of oocytes from *stimulated* cycles | *H19* | ↑ |
| Khoueiry *et al.* (2008) | Human | | Medicult supplemented with FSH, hCG and 10% patient serum | Rescue IVM of oocytes from *stimulated* cycles | *KCNQ1OT1* (*KvDMR1*) | ↓ |

=, unchanged; ↑, increased; ↓, decreased DNA methylation compared with *in vivo* grown oocytes.

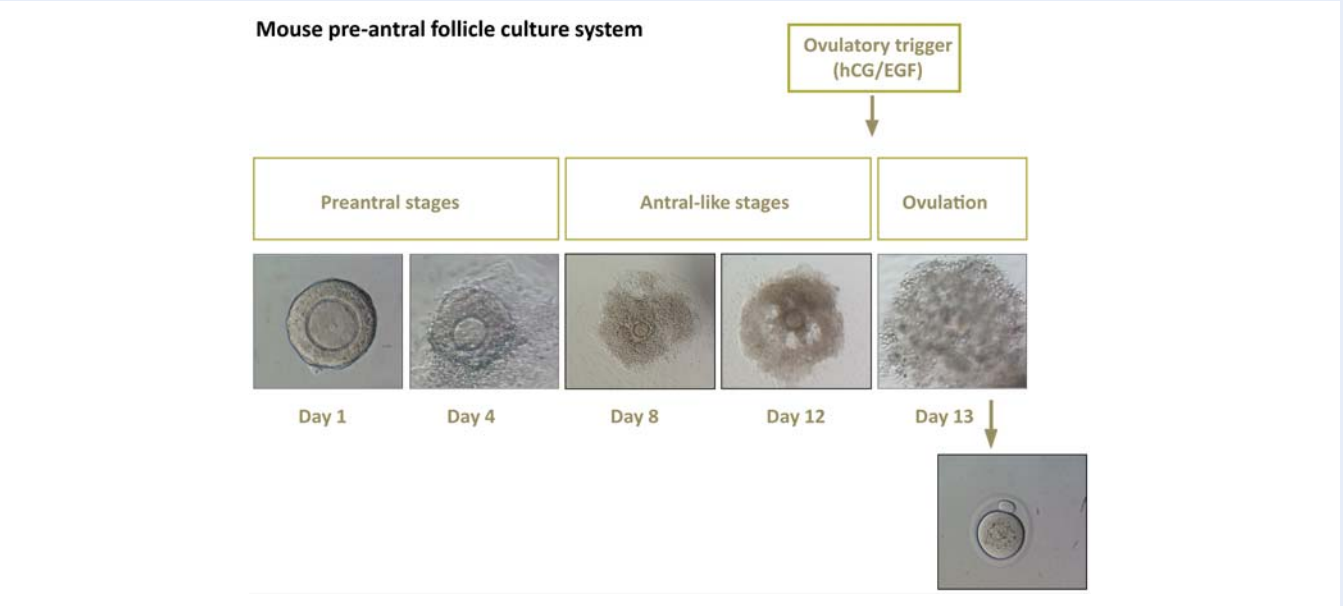


**Figure 4** Representative overview of the mouse *in vitro* follicle culture system in defined conditions. Day 1: early pre-antral follicle. The pre-antral follicle is attached to the bottom of the culture dish (Day 1–4) by the proliferating theca cells; the granulosa cells break through the basal membrane (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/epidermal growth factor stimulus on these follicles provokes the release of a mucified cumulus–corona complex (Day 13) surrounding a MII oocyte. Note that the oocyte diameter increases from 50–55 μm (diameter in early pre-antral follicle) up to 72–75 μm (diameter before ovulation) (Cortvrindt and Smitz, 2002).

Lucifero *et al.*, 2002, 2004; Hiura *et al.*, 2006). This demonstrates that imprints for these genes are correctly established under the *in vitro* follicle culture conditions (Anckaert *et al.*, 2009a).

These results were later confirmed in a study which combined bisulphite sequencing with the so-called LD technique to increase the sensitivity for detection of DNA strands with aberrant methylation patterns in pooled oocyte samples (Trapphoff *et al.*, 2010). Using the same mouse strain and relying on the same *in vitro* follicle culture system, this group showed that the frequency of abnormal methylation, overall or at individual CpG sites, was not significantly different for *H19*, *Snrpn* and *Igf2r* in fully grown GV oocytes that had developed *in vitro* or had grown *in vivo* (collected as GV stage oocytes from large antral follicles in unstimulated cycles). Moreover, a very recent paper from the same group demonstrated that 2-cell embryos obtained after *in vitro* fertilization of oocytes derived from this *in vitro* follicle system yielded similar DNA methylation patterns at *H19*, *Snrpn* and *Igf2r* as *in vivo* produced controls from unstimulated mice (El Hajj *et al.*, 2011).

Finally, these results were in accordance with a study of oocytes obtained after a 12 day *in vitro* follicle culture from early secondary follicles of 12-day-old CD-1 mice (Shen *et al.*, 2007). The *Igf2r* DMR analysis revealed a 92.2% methylation at potential CpG-sites (similar to 96.3% in control oocytes). However, in this study, the DNA methylation analysis was not the primary aim of the study and consequently, the conclusions were based on a low number of repeats (Shen *et al.*, 2007).

### *Mechanistic studies using mouse* in vitro *follicle culture*

*High recombinant FSH levels in culture medium.* The mouse follicle culture system with proved normal imprinting establishment under defined conditions was subsequently used as a bioassay to study the influence of several suboptimal culture conditions on imprinting establishment in oocytes.

Conflicting data have been reported concerning the effect of ovulation induction on imprinting establishment in mouse oocytes. In a first study, BDF and ICR mice were superovulated with high doses of equine chorionic gonadotropin (eCG) (7.5 IU eCG for 3 days), followed by an injection with 5 IU hCG 24 h later (Sato *et al.*, 2007). In superovulated mouse oocytes, the authors found no differences in methylation for *Mest*, *Lit1* and *Zac*, but for *H19* aberrant hypermethylated clones were more frequent compared with oocytes from unstimulated antral follicles (Sato *et al.*, 2007).

However, a recent study found normal imprinted DNA methylation at *Snrpn*, *Peg3* and *H19* in superovulated oocytes from B6(CAST7p6) × B6 mice, superovulated with a single dose of 6.25 or 10 IU of eCG, followed by the same dose of hCG 46–48 h later (Denomme *et al.*, 2011). The bisulphite sequencing technique was applied to single oocytes embedded in agarose beads and an elegant technique was applied to exclude somatic cell contamination. In line with the latter study, a recent study based on the LD technique found no alterations in imprinted DNA methylation for *H19*, *Snprn* and *Igf2r* in 2-cell embryos derived from superovulated C57BL/6J x CBA/Ca mice (El Hajj *et al.*, 2011). The contradictory findings between the latter two studies and the former one might be due to the fact that higher eCG doses over a longer treatment interval were applied in Sato's study or to the fact that different mouse strains were used. Previous work on *in vitro* cultured mouse blastocysts has shown that some mouse strains might be more susceptible to culture-induced imprinting defects than others (Doherty *et al.*, 2000). Alternatively, somatic cell contamination should always be considered as an eventual pitfall in case of discordant findings.

A supraphysiological dose of 100 IU/L of r-FSH was used during follicle culture, which exceeds by a factor of 20 the minimal needs for maximal follicle survival and MII rate (Adriaens *et al.*, 2004). However, no effects on DNA methylation levels at regulatory sequences of *Snrpn*, *Igf2r* and *H19* could be detected in MII oocytes, suggesting that high doses of FSH do not interfere with normal imprinting establishment (Anckaert *et al.*, 2009a). These results are in line with two *in vivo* studies in mouse showing that ovulation induction with eCG does not interfere with oocyte imprinting establishment (Denomme *et al.*, 2011; El Hajj *et al.*, 2011).

*High ammonia levels in culture medium and mineral oil overlay.* Ammonium accumulates in cell culture medium due to ammonia release from amino acid metabolism and due to the chemical decomposition of amino acids in culture medium incubated at 37°C (Schneider *et al.*, 1996). The addition of ammonium to culture medium during mouse preimplantation embryo culture led to increased expression of the imprinted H19 gene (Lane and Gardner, 2003).

Oil overlay is widely used in IVM, although it has been associated with delayed nuclear maturation and reduced developmental capacity in pig IVM (Shimada *et al.*, 2002) and with delayed meiosis I progression in mouse oocytes after *in vitro* follicle culture (Segers *et al.*, 2008). Application of mineral oil overlay to the follicle culture system leads to a reduction of more than 50% in steroid hormone levels (Miller and Pursel, 1987; Anckaert *et al.*, 2010). Reduced steroid hormone levels may pose a threat to normal imprinting establishment by increasing the availability of unbound steroid hormone receptors for xenobiotic compounds. It has been shown that during critical periods of mammalian development, xenobiotic compounds with estrogenic effects, such as bisphenol A, may alter DNA methylation patterns (Ho *et al.*, 2006).

The findings of normal imprinting establishment in oocytes derived from an *in vitro* follicle culture system (Anckaert *et al.*, 2009a) were in contrast with another study, suggesting that *in vitro* follicle culture can lead to aberrant imprinting in fully grown GV mouse oocytes (Kerjean *et al.*, 2003). In Kerjean's study, follicle culture was performed in small culture medium volume droplets under a thick mineral oil layer, yielding 10-fold higher ammonia levels in culture medium than the *in vitro* follicle culture system allowing normal imprinting establishment (Anckaert *et al.*, 2009b). However, the addition of ammonium acetate and a mineral oil overlay in the latter system did not affect follicle survival, MII rate and/or MII oocyte diameter and normal DNA methylation patterns at *Snrpn*, *Igf2r* and *H19*, demonstrating that ammonium accumulation and mineral overlay during follicle culture do not induce aberrant imprinting establishment at the studied regulatory sequences in MII oocytes (Anckaert *et al.*, 2009b).

*Low methyl donor levels in culture medium.* The methionine metabolic pathway plays an important role in DNA methylation processes. The essential amino-acid methionine is actively transported into oocytes and converted into S-adenosylmethionine (Menezo *et al.*, 1989), the sole methyldonor for DNA methylation reactions. Vitamin B12, folic acid, choline and vitamin B6 may also affect DNA methylation levels through their involvement in the methionine cycle (Van den Veyver, 2002).

Several studies in mouse have shown that maternal dietary methyl donor levels might influence DNA methylation levels in the offspring (Waterland and Jirtle, 2003; Waterland *et al.*, 2006; Sinclair *et al.*, 2007). Commercially available embryo culture media feature an important variation in the levels of these methyl donors (Steele *et al.*, 2005). Low methyl donor levels during *in vitro* follicle culture in mouse led to a dramatic decrease in polar body extrusion rate, but no alterations in DNA methylation at *Snrpn*, *Igf2r* and *H19* were found (Anckaert *et al.*, 2010). However, for *Mest* DMR, a slight reduction in DNA methylation was found compared with control follicle culture conditions. The changes were not due to aberrant methylation of the entire allele, but were located at specific individual CpG sites in the affected *Mest* alleles. The biological significance of single or few CpG mutations is currently unknown, but the overall density rather than individual CpG methylation is probably involved in regulating imprinted gene expression (Sontag *et al.*, 2006). Methionine levels in culture medium were lowest at the final days of follicle culture (when *Mest* acquires DNA methylation, see Fig. 2), probably due to a consumption of the amino acid with increasing follicle growth; and this might explain the increased susceptibility of *Mest* (Anckaert *et al.*, 2010).

### *Ovine* in vitro *follicle culture*

There are only limited data on oocyte imprinting establishment during follicle culture in other animal species. In sheep, a 14-day *in vitro* pre-

antral follicle culture system (using follicles from prepubertal animals) was used to demonstrate that oocytes from early antral follicles yielded similar methylation patterns at *H19* and *IGF2R* as oocytes at similar stages grown *in vivo* and collected in stimulated (Barboni *et al.*, 2011) or unstimulated (Colosimo *et al.*, 2009) cycles.

## Ovarian tissue culture in mouse

A few research groups have examined imprinting establishment in oocytes derived from ovarian tissue culture.

E15.5 embryonic ovary fragments from Swiss TO mice were cultured for 3 weeks in Waymouth medium with serum (Lees-Murdock *et al.*, 2008). Some oocytes reached full size (70 μM), but no secondary follicles were formed, most likely due to the absence of formation of a theca cell layer. Some DNA methylation occurred for *Snrpn* and *Igf2r*, while no methylation occurred for *Mest* (which can be explained by the fact that the latter becomes methylated later than *Snrpn* and *Igf2r* during *in vivo* oocyte growth). These results suggest that complete methylation at maternally methylated imprinted genes in oocytes probably requires progression to the secondary follicle stage. No aberrant hypermethylation of *H19* occurred in oocytes during the *in vitro* culture.

Song *et al.* (2009) cultured 12.5 dpc fetal Kunming mouse ovaries *in vitro* for 28 days. GV stage oocytes were obtained reaching a diameter of more than 70 μM, but the number of granulosa cells was lower than *in vivo* and no antral follicle formation occurred. Oocyte DNA methylation at *Igf2r* and *Peg3* occurred during the *in vitro* culture, although at a slower rate than for comparable *in vivo* stages from unstimulated mice.

In another study, 12.5 dpc fetal mouse ovaries (C57BL/6J x CBA) were cultured *in vitro* for 17 days in Waymouth medium (Obata *et al.*, 2002). These ovaries contained many secondary follicles that were isolated and cultured for a further 11 days. Some follicles showed antrum formation at the end of culture and the oocyte diameter reached 64 μM. DNA methylation patterns at *Igf2r* at each stage of culture were similar to these from *in vivo* grown oocytes of the same stage. Furthermore, transfer of the nuclei of these cultured oocytes into enucleated fully grown oocytes from adult mice made them competent to resume meiosis and after serial nuclear transfer and IVF, normal appearing live offspring could be obtained. DNA methylation analysis of kidney tissue in the offspring revealed normal imprinted methylation of *Igf2r*, *Snrpn* and *Mest*. The latter study suggests that correct imprinting establishment can be obtained in mouse oocytes derived from a two-step procedure involving fetal ovarian tissue culture followed by *in vitro* follicle culture from the pre-antral stage onwards. However, the DNA methylation analysis of oocytes was not the primary aim of the study and therefore, the conclusions are based on a lower number of repeats.

## Oocyte IVM and oocyte imprint establishment in animal models

### *Bovine and ovine IVM*

In large animal models, IVM involves the submission of GV stage oocytes to 24 h of culture in a maturation environment to obtain fertilizable MII oocytes.

Using the LD bisulphite sequencing technique (on pools containing 10 oocytes) which allowed the amplification of a high number of alleles, Heinzmann could demonstrate that bovine IVM in either modified synthetic oviduct fluid or in Tissue Culture Medium 199 did not significantly alter imprinted DNA methylation at *H19*, *PEG3* and *SNRPN* when compared with *in vivo* matured superovulated oocytes (Heinzmann *et al.*, 2011).

A recent study in sheep confirmed normal imprinted DNA methylation in IVM oocytes: in a small number of *in vitro* matured MII oocytes, only unmethylated alleles for *H19* DMR and only hypermethylated alleles for *IGF2R* DMR2 were present, similar to patterns found in *in vivo* grown oocytes from medium antral follicles obtained from unstimulated animals (Colosimo *et al.*, 2009).

### *Mouse postovulatory oocyte aging* in vitro

There are no studies in rodents on imprinting establishment of fully grown GV stage oocytes matured to the MII stage in culture (IVM), but two groups have examined the effect of *in vitro* ageing of mouse MII oocytes recovered after ovarian stimulation.

Imamura studied DNA methylation at *Mest* in adult C57BL/6 x CBA mouse MII oocytes after eCG/hCG ovulation induction and *in vitro* culture (Imamura *et al.*, 2005). In MII oocytes collected 14 h post-hCG, imprinted methylation was not yet fully established. This was in contrast with another study in a different mouse strain (CD-1) showing a full methylation at *Mest* in MII oocytes recovered after low-dose ovulation induction (Lucifero *et al.*, 2002). The discordant findings between the latter two studies may be due to the fact that different regions of the *Mest* DMR were amplified, the use of different mouse strains or to a somatic cell contamination in Imamura's study. After culture of MII oocytes in M16 medium for 8 h (corresponding to 22 h after hCG), only methylated alleles were found, suggesting a possible *de novo* methylation at *Mest* in MII oocytes during *in vitro* culture. When MII oocytes were aged *in vitro* for 28 h (corresponding to 42 h post-hCG), unmethylated alleles were again observed, implying that imprinted DNA methylation at the studied region is unstable and might be affected by oocyte aging during *in vitro* culture.

*In vivo*, mouse oocytes are ovulated in the MII stage ~12 h after the LH surge and are expected to be fertilized within 6 h after ovulation (Braden and Austin, 1954). Outside this window, postovulatory oocyte aging occurs. However, the *in vitro* ageing applied in Imamura's study was excessively long, considering the fact that already at 16 h after maturation stimulus, a maximum proportion of *in vitro* grown mouse oocytes reach maturation, and that at 21 h post-hCG aging effects are visible on the spindle apparatus (Segers *et al.*, 2008). Furthermore, the cultured MII oocytes were derived from superovulated cycles and although two well-designed mouse studies found no evidence of alterations in imprinting establishment in superovulated oocytes (Denomme *et al.*, 2011; El Hajj *et al.*, 2011), another study in mice suggested that some imprinted genes might be affected by ovulation induction (Sato *et al.*, 2007).

Another study examined the effect of postovulatory oocyte aging on imprinted DNA methylation at *Snrpn* and *Mest* in mouse oocytes (Liang *et al.*, 2008). Oocytes were aged *in vivo* (up to 29 h after hCG) and *in vitro* (collected 13 h after hCG and cultured *in vitro* for up to 16 h, corresponding to 29 h post-hCG *in vivo*). *In vitro* culture was performed for cumulus–oocyte complexes (COCs) and for denuded oocytes. For *Mest* no alterations were found. However, for *Snrpn* bisulphite sequencing showed some demethylated clones for the 29 h post-hCG *in vivo* aging condition and after *in vitro* aging

(29 h post-hCG) but only in the case of denuded oocytes. Possible limitations of this study were that only two batches of oocytes were tested and that acid M2 was used for removal of the zona and attached cumulus cells. The author's laboratory has the experience that using acid tyrode to remove the zona with attached cumulus cells can lead to somatic cell DNA contamination (unpublished results). Acid tyrode probably lyses the cumulus cells attached to the zona, releasing DNA that apparently may not be fully removed despite repeated washing steps.

However, the importance of cumulus cells for oocyte maturation is well known and therefore the effect of cumulus cells on imprinted DNA methylation in cultured oocytes should be re-examined in future studies to allow definitive conclusions.

### Human studies: imprint establishment in *in vitro* matured oocytes obtained from stimulated cycles

Only a single research team has studied DNA methylation at two imprinted genes in human IVM oocytes. However, these studies have been performed on low-quality oocytes from regular stimulated cycles for IVF/ICSI, which failed to respond to maturation after the standard hCG stimulus.

In a first study, DNA methylation at *H19* DMR was studied (Borghol *et al.*, 2006). Immature oocytes from stimulated cycles were retrieved and *in vitro* matured for 24 h in Medicult maturing medium, supplemented with FSH, hCG and 10% patient serum. Oocytes were examined at the GV stage after retrieval (D0) and at the GV, MI and MII stage after IVM. For the GV oocytes at D0, all clones showed an unmethylated pattern as expected in two replicates tested. After ovarian stimulation followed by IVM, a high frequency of aberrant methylation was recorded in GV and MI oocytes (6 out of 11 pools), and in MII oocytes abnormalities were found in two out of nine replicates. These results suggest that ovarian stimulation followed by IVM might lead to aberrant imprinting at *H19* (particularly in MI-blocked oocytes).

Loss of methylation on the maternal allele of the *KCNQ1OT1* gene (KvDMR1 region) has been associated with BWS after ART conception (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Halliday *et al.*, 2004; Chang *et al.*, 2005; Rossignol *et al.*, 2006; Sutcliffe *et al.*, 2006; Bowdin *et al.*, 2007; Gomes *et al.*, 2007). The same group examined DNA methylation at the maternally methylated KvDMR1 region in human oocytes after ovarian stimulation and IVM (Khoueiry *et al.*, 2008). Immature oocytes (GV or MI) and MII oocytes after standard ovarian stimulation were collected for either direct analysis or for IVM (GV and MI) during 28 h. The percentage of methylated alleles was, respectively, 60.4, 62.5 and 89.5% for fully grown GV, MI and MII oocytes retrieved after ovarian stimulation, suggesting that KvDMR1 has not acquired full imprinting establishment in GV oocytes and also suggesting methylation advances at this region with meiosis progression, at least in oocytes collected from stimulated cycles. These results were in conflict with another study that showed a fully methylated pattern in human GV oocytes from ART cycles for a region of KvDMR1 located downstream from the region studied by Khoueiry (Geuns *et al.*, 2007) and also in conflict with mouse data showing a full methylation of KvDMR1 in oocytes from early antral follicles (Hiura *et al.*, 2006). After 28 h of IVM, oocytes that became MII showed somewhat lower methylation levels than the MII oocytes obtained 36 h after hCG (78.3 versus 89.5%, $P < 0.01$).

Oocytes were also retrieved from natural cycles in PCOS patients and subjected to IVM, where GV and MI-arrested oocytes were available for analysis. Compared with the same stage oocytes after ovarian stimulation and IVM (whole group and PCOS-group), GV and MI oocytes obtained in PCOS patients from natural cycles and subsequently *in vitro* matured displayed a slightly (around 6%) higher methylation at KvDMR1, suggesting that ovarian stimulation might interfere with imprinting establishment at the studied region. The authors conclude that ovarian stimulation might recruit immature oocytes that are unable to complete imprinting establishment during the short IVM period, although culture-induced effects could not be excluded.

In a third study from the same group, immature oocytes were retrieved after ovarian stimulation to be either *in vitro* matured directly or to be vitrified at the GV stage and subsequently *in vitro* matured (Al-Khtib *et al.*, 2011). Oocyte vitrification did not alter the methylation status of the imprinted *H19* and *KCNQ1OT1* genes. Moreover, and in contrast to the two previous studies from the same group, the IVM condition induced less alterations in imprinted DNA methylation for the two genes: 3 out of 34 sequenced clones showed a gain of methylation for H19 in 48 MII oocytes, and 2 out of 37 sequenced clones showed a loss of methylation at *KCNQ1OT1* in 20 MII oocytes. The IVM duration was extended from 26–28 to 36 h in this study. Whether the extension of IVM duration might explain the lower occurrence of imprinting errors in the latter study remains to be determined. It seems unlikely, however, that extended culture would reduce the extent of aberrant hypermethylation at H19.

## Discussion

### Current data on imprinting establishment from animal models

The bovine IVM and mouse *in vitro* follicle culture models provide reassuring data on imprinted DNA methylation acquisition in oocytes (Anckaert *et al.*, 2009a; Trapphoff *et al.*, 2010; El Hajj *et al.*, 2011; Heinzmann *et al.*, 2011).

The use of serum in culture medium has been implicated in aberrant imprinting in preimplantation embryo culture. In sheep and cattle, culture of preimplantation embryos frequently leads to the so-called large offspring syndrome (LOS), which is characterized by overgrowth and developmental abnormalities during fetal and post-natal development (which are reminiscent of BWS in human). In sheep, *in vitro* culture until the blastocyst stage, followed by transfer into recipient females, leads to fetuses that feature a strong reduction in DNA methylation levels at the *IGF2R* ICR correlating with a loss in *IGF2R* expression (Young *et al.*, 2001). Ovine studies have shown that the presence of serum in culture medium can lead to LOS (Sinclair *et al.*, 1999). Likewise, aberrant imprinting has been described after *in vitro* embryo culture in mouse. Decreased expression of the *H19* and *Igf2* genes has been described in mice fetuses after blastocyst culture in M16 medium supplemented with fetal calf serum (Khosla *et al.*, 2001). In contrast, the use of fetal calf serum in the mouse follicle culture system does not interfere with imprinted DNA methylation (Anckaert *et al.*, 2009a; Trapphoff *et al.*, 2010; El Hajj *et al.*, 2011). Furthermore, the correct imprinting establishment in mouse

oocytes after *in vitro* follicle culture under various treatment and (sub-optimal) follicle culture conditions such as high doses of r-FSH, mineral oil overlay, ammonia accumulation and low methyl donor levels suggests that DNA methylation establishment at regulatory sequences of imprinted genes in oocytes is a robust process (Anckaert *et al.*, 2009a, b, 2010), whereas the preimplantation embryo appears to be susceptible to culture-induced aberrant imprinting maintenance. The former is also illustrated by the finding of correct imprinting establishment in mouse oocytes derived from fetal ovarian tissue culture followed by *in vitro* follicle culture from the pre-antral stage onwards (Obata *et al.*, 2002).

In contrast, Kerjean found that *in vitro* follicle culture can lead to aberrant imprinting in fully grown GV mouse oocytes (Kerjean *et al.*, 2003), especially at the *Igf2r* locus. The mouse strain, the culture medium used and the early pre-antral follicle stage at the start of the culture were similar to the studies cited above (Anckaert *et al.*, 2009a, b, 2010; Trapphoff *et al.*, 2010; El Hajj *et al.*, 2011). However, in Kerjean's study follicle culture was performed in small culture volume droplets under mineral oil, possibly leading to the accumulation of some metabolites that might influence imprinting establishment. Therefore, continuing research is necessary to identify possible risk factors and procedures.

Another possible explanation for the discordant findings is that different regions were studied by these groups, e.g. for the *H19* gene: respectively, the CTCF1-2 region (Anckaert) and the CTCF3–4 region (Kerjean *et al.*, 2003). The *H19* CTCF1–2 region was shown to be more susceptible than the CTCF3–4 region to aberrant DNA methylation after IVF and embryo culture in mouse (Fauque *et al.*, 2007), suggesting then, however, that the *H19* region studied in our experiments might be more susceptible to culture-induced effects than the region studied by Kerjean. The possibility of somatic cell contamination should also be kept in mind as a cause of the findings in Kerjean's study.

Bovine IVM did not significantly alter imprinted DNA methylation at *H19*, *PEG3* and *SNRPN* when compared with *in vivo* maturation (Heinzmann *et al.*, 2011).

However, mRNA expression of the three imprinted genes was up-regulated in bovine IVM oocytes, suggesting that a regulatory mechanism other than DNA methylation might be affected by the IVM conditions (Katz-Jaffe *et al.*, 2009; Heinzmann *et al.*, 2011). DNA methylation is an essential feature, but not the only component of imprinting as other epigenetic mechanisms such as histone tail modifications (e.g. methylation and acetylation) and microRNAs, play a role in regulating genomic imprinting.

In rhesus monkey, a cDNA array-based analysis showed an overexpression of *MEST* and *PLAGL1*, two maternally imprinted genes, in *in vitro* matured oocytes, indicating a possible deregulation of genome imprinting after IVM, although DNA methylation (and other epigenetic features regulating genomic imprinting) were not examined in that study (Lee *et al.*, 2008).

More studies are therefore necessary to determine the possible influence of *in vitro* culture of oocytes on epigenetic modifications (other than DNA methylation) regulating genomic imprinting. Finally, well-designed studies should be performed to assess whether *in vitro* oocyte aging might affect imprinted DNA methylation in oocytes as suggested by two studies (Imamura *et al.*, 2005; Liang *et al.*, 2008).

## Imprinting maintenance

To allow full-term development, not only should imprinted DNA methylation be accurately established during gametogenesis, but equally important is the correct maintenance of the germline DNA methylation patterns, despite genome-wide changes in DNA methylation during preimplantation. However, no data are currently available on the effect of oocyte culture on imprinted DNA methylation maintenance during preimplantation development.

After fertilization, a wave of DNA demethylation occurs in the preimplantation embryo. In mouse, the paternal genome undergoes a rapid DNA demethylation that is completed within 6 h after fertilization, suggesting an active (enzymatic) process (Santos *et al.*, 2002). In contrast, the step-wise demethylation of the maternal genome is thought to be a passive process in absence of DNA methylation maintenance during cell divisions until the blastocyst stage (Rougier *et al.*, 1998; Santos *et al.*, 2002). Around the time of implantation, there is a wave of *de novo* DNA methylation resulting in a highly methylated inner cell mass and a less methylated trophectoderm (reviewed in Feil, 2009). The active demethylation of the paternal genome has also been described in the human; passive demethylation and *de novo* methylation are also functionally conserved between species, but the timing and the extent of (de)methylation varies between species (reviewed in Dean *et al.*, 2005).

Certain DNA sequences, such as ICRs of imprinted genes are presumed to be resistant to the genome-wide changes in DNA methylation after fertilization, although ICRs are not fully protected so that some dynamic changes in allele methylation occur during preimplantation development, resulting in some size variation between gametic and embryonic DMRs (Tomizawa *et al.*, 2011).

A number of protein factors have been discovered to play a role in the maintenance of imprinting. Dnmt1o is a truncated form of the maintenance Dnmt1, which is specifically expressed and stored in oocytes. Although the exact mechanism is unknown and somewhat controversial, maternal Dnmt1o and zygotic Dnmt1s appear to cooperate to maintain imprinted methylation in the preimplantation embryo (reviewed in Weaver *et al.*, 2009). The absence of Dnmt1 in the early embryo leads to a loss of DNA methylation at paternally and maternally methylated genes resulting in embryonic death (Hirasawa *et al.*, 2008). Several other *trans*-acting factors expressed in the oocyte have been implicated in maintenance of imprinting during preimplantation development such as Zfp57, Stella and Mbd3 (Nakamura *et al.*, 2007; Reese *et al.*, 2007; Li *et al.*, 2008). The role of Zfp57 in DNA methylation maintenance is conserved between mice and human as shown in transient neonatal diabetes caused by autosomal recessive *ZFP57* mutations and featuring a loss of methylation at several DMRs including *PLAGL1* (Mackay *et al.*, 2008).

An up-regulation of the maintenance Dnmt1 has been shown in bovine IVM oocytes, but the functional consequences are currently unknown (Heinzmann *et al.*, 2011) and more studies are therefore needed to determine whether the expression of DNA maintenance factors is altered by *in vitro* oocyte culture.

A number of mouse studies have suggested that ovulation induction might interfere with imprinting maintenance after fertilization. Ovulation induction of mice led to a higher proportion of blastocysts without detectable *H19* expression compared with controls (Fauque *et al.*, 2007) and resulted in aberrant biallelic expression of *Snrpn*

and *H19* in placentas (Fortier *et al.*, 2008). The expression of these genes was not altered in embryos in the latter study, confirming an earlier study suggesting that trophectoderm tissues might be more susceptible to aberrant imprinting induced by ART than the embryo proper (Mann *et al.*, 2004). A recent study examined the effect of ovulation induction on DNA methylation in individual mouse blastocysts obtained from superovulated C57BL/6 (CAST7) females mated with C57BL/6 males (Market-Velker *et al.*, 2010b). Ovulation induction resulted in a dose-dependent loss of methylation at the maternally methylated *Snrpn*, *Peg3* and *Kcnq1ot1* loci; and a dose-dependent gain at the paternally methylated *H19*. In contrast, DNA methylation establishment in oocytes from C57BL/6 (CAST7) mice was not affected by conventional and high eCG/hCG doses (Denomme *et al.*, 2011).

Collectively, these *in vivo* studies suggest that ovulation induction might interfere with the capacity of oocytes to maintain imprinting during preimplantation development. Therefore, it remains to be determined whether oocyte culture might also affect imprinting maintenance after fertilization rather than imprinting establishment during oogenesis.

## Current data from human studies

### *Validity of animal models for human*

Studies on imprinting establishment in human oocytes have been performed in stimulated ART cycles. Two studies have shown conflicting results for the timing of imprinting establishment at the *SNURF-SNRPN* locus, which is involved in the PWS and AS, with methylation acquisition complete after fertilization (El-Maarri *et al.*, 2001) or in GV oocytes (Geuns *et al.*, 2003). DNA methylation was found to be already established in GV stage oocytes for the *KvDMR1* also (Geuns *et al.*, 2007). In contrast, Khoueiry found hypermethylated alleles at *KvDMR1* in only two-thirds of fully grown GV oocytes and an increase in methylation with meiotic progression (Khoueiry *et al.*, 2008).

In only two studies, human oocytes from unstimulated cycles have been examined. Sato found a hypermethylated pattern in fully grown GV oocytes from antral follicles (obtained in ovarian biopsy samples) for *MEST*, *LIT1* and *ZAC* DMRs (Sato *et al.*, 2007). Finally, Arima described acquisition of full DNA methylation for *HYMAI/PLAGL1* (*ZAC*) DMR already at the pre-antral follicle stage (Arima and Wake, 2006).

The majority of studies therefore suggest that imprinting is established in human oocytes before fertilization as in mouse, suggesting that the mouse is a good model for the study of imprinting establishment during oocyte culture.

However, it should be considered that some differences are present between species, e.g. some genes such as *Igf2r* are imprinted in mouse but not in human; and expression of Dnmt3L, which is indispensable for imprinting establishment in mouse oocytes and is also present in growing bovine oocytes (O'Doherty *et al.*, 2012) was only detected after fertilization in human (Huntriss *et al.*, 2004). Furthermore, in mouse, the maximal oocyte diameter and the process of chromatin compaction (non-surrounded nucleolus to fully surrounded stage) are reached when the antrum is formed (Mattson and Albertini, 1990). The mouse oocyte is transcriptionally silent when maturation starts and has all the proteins needed to resume meiosis. In contrast, in larger mammals, such as cow and human, the oocyte still grows in the antral follicle and transcription is needed during final meiotic maturation in order to reach the MII stage (Bilodeau-Goeseels, 2011). Consequently, extrapolating from mouse to human may imply a risk, thus the bovine IVM model provides additional valuable information.

### *IVM, a technique prone to imprinting errors in human?*

IVM may be defined as the IVM of oocytes from COCs out of small antral follicles with a diameter $\leq$10 mm, from cycles with leading follicles not exceeding a diameter of 12 mm (Son *el al.*, 2008). However, results are confounded by huge differences in the type of methodology used clinically (Nogueira *et al.*, 2008; Sirard, 2011; Smitz *et al.*, 2011).

The final stages of cytoplasmic maturation, essential for developmental competence of the oocyte, take place in the follicles recruited by the intercycle FSH rise. A normal follicular phase lasts 10–12 days (Gougeon, 1986); it remains to be studied whether taking out COCs from small follicles around Day 7 or 8 after menses would compromise the normal imprinting pattern. This question is relevant, as the meiotic maturation timespan after retrieving an oocyte from its follicle environment is considerably shortened by a few hours compared with that *in vivo* after a positive maturation stimulus (Albuz *et al.*, 2010).

Studies have suggested an increased incidence in rare human imprinting disorders such as BWS in children conceived after ART (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Halliday *et al.*, 2004; Chang *et al.*, 2005; Rossignol *et al.*, 2006; Sutcliffe *et al.*, 2006; Bowdin *et al.*, 2007; Gomes *et al.*, 2007; Lim *et al.*, 2009), although the reported incidence of BWS remains extremely low and was not confirmed in other studies (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007). There is currently no evidence that IVM is associated with an increased risk for congenital malformations, abnormal fetal and neonatal growth or imprinting syndromes, but there are currently only limited data on the safety of IVM as only slightly more than 1000 births have been reported worldwide.

Studies of human oocytes after ovarian stimulation followed by IVM found aberrant DNA methylation at *H19* and KvDMR1 (Borghol *et al.*, 2006; Khoueiry *et al.*, 2008; Al-Khtib *et al.*, 2011). However, it is not clear from these studies whether IVM by itself should be considered causal for the aberrant imprinting. First, it was not excluded that ovarian stimulation interferes with imprinting establishment in human oocytes. Sato studied DNA methylation patterns at *MEST*, *LIT1*, *ZAC* and *H19* in stimulated oocytes and in oocytes obtained from naturally cycling ovaries (Sato *et al.*, 2007). In GV and MI oocytes retrieved after ovarian stimulation (and analysed by single-cell bisulphite PCR), an unmethylated allele for *MEST* and a hypermethylated allele for *H19* was found in, respectively, 6 out of 16 and in 2 out of 6 examined oocytes. The authors conclude that although the DNA methylation changes observed in human stimulated oocytes may be due to the underlying infertility or the advanced maternal age, ovarian stimulation might be (at least partly) responsible for the observed aberrant imprinting in oocytes. Further studies on imprinting establishment in human oocytes are mandatory to reach definitive conclusions.

There were a number of other important limitations and confounding factors in the human IVM studies such as the use of low-quality oocytes that failed to respond to ovarian stimulation, the underlying infertility and the advanced maternal age. Indeed, a Dutch study found an increased incidence for imprinting disorders in children

from couples with fertility problems (time to pregnancy >12 months) (Doornbos *et al.*, 2007). Moreover, aberrant methylation at maternally and paternally methylated ICRs has been reported in association with poor semen parameters or male infertility, correlating with the severity of oligozoospermia (Marques *et al.*, 2004, 2008; Kobayashi *et al.*, 2007; Poplinski *et al.*, 2010).

Also, in the IVM studies, the IVM period applied was short: 24–28 h compared with 30–36 h for *in vivo* maturation and for other IVM protocols used in clinical practice. Although 24–28 h cultures are often used in human IVM given it appears sufficient to reach MII, the accelerated kinetic could influence imprinting establishment.

Finally, the fact that the *in vitro* matured oocytes had no or few cumulus cells attached (which play a major role in oocyte maturation) may be an important limitation. The importance of the somatic environment was illustrated in an *in vivo* study with sex-reversed mouse germ cells suggesting that the somatic environment of the female germline contributes to the imprinting establishment as the female imprinting patterns for *Peg3* were dependent on the response of germ cells to undergo oogenesis, but not on their sex chromosome constitution (Durcova-Hills *et al.*, 2006).

### Conclusion

Animal models provide reassuring data on imprinted DNA methylation acquisition in cultured oocytes. Using a mouse *in vitro* follicle culture system, influences of treatment and suboptimal culture conditions were found to have no or only minor effects.

Nevertheless, additional studies are needed to investigate whether the expression and DNA methylation of imprinted genes in blastocysts, fetuses and placental tissue derived from oocytes obtained after IVM and follicle culture is unaltered, to show that: (i) other epigenetic modifications (besides DNA methylation) regulating genomic imprinting are not altered by the *in vitro* culture conditions; and (ii) *in vitro* culture does not cause a disruption of maternal-effect gene products subsequently required for genomic imprint maintenance during preimplantation development.

Although animal models provide reassurance, no definitive conclusion on normal imprinting establishment in human IVM oocytes can be drawn as well-designed human studies are currently not available. Optimized IVM procedures currently under development will require assessment in donated oocytes from young, fertile healthy females (not exposed to ovarian stimulation) to exclude possible confounding factors. Equally important is the use of appropriate techniques to assess DNA methylation such as LD bisulphite sequencing providing increased sensitivity and reduced risk for amplification/cloning bias. Finally, comparative analysis of epigenetic patterns in cord blood and placenta from children born from either IVM pregnancies or spontaneous pregnancies, will allow a more detailed insight into the influence of IVM on the epigenome and to draw definitive conclusions on the epigenetic safety of human IVM.

## Authors' roles

E.A. conceptualized the review, performed the systematic literature search, the data extraction and interpretation and wrote the review. M.D.R and J.S. revised the paper. All the authors approved the final version of the manuscript.

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## Conflict of interest

None declared.

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