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Notes

Dynamic CpG methylation of the *KCNQ1OT1* gene during maturation of human oocytes

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ABSTRACT

Background: Imprinted genes, many of which are involved in development, are marked during gametogenesis to allow their parent-of-origin specific expression, and DNA methylation at CpG islands is part of this epigenetic mark. Maternal imprint is apposed on oocyte during growth and maturation. Factors interfering with normal oocyte differentiation such as gonadotrophin stimulation and in vitro maturation (IVM) may possibly alter imprint resetting.

Methods: We examined the methylation of the KCNQ1OT1 differentially methylated region (KvDMR1) in human oocytes at different stages of their development: germinal vesicle (GV), metaphase I (MI) or metaphase II (MII).

Results: About 60% of alleles were fully methylated in GV oocytes and that full imprint is acquired in most MII oocytes. Similarly to in vivo, de novo methylation of DNA occurred in vitro during oocyte maturation. Following in vitro culture for 28 h, GV and MI oocytes are significantly more methylated when they are obtained from natural cycles than from patients undergoing gonadotrophin stimulation.

Conclusion: This observation suggests that hyperstimulation likely recruits young follicles that are unable to acquire imprint at KvDMR1 during the course of the maturing process.

Mammalian oocytes are arrested at prophase of the first meiotic division before induction of maturation by the preovulatory luteinising hormone surge. Oocyte maturation corresponds to the re-initiation and completion of the first meiotic division, with progression from the diplotene to the metaphase II stage. It is characterised by disappearance of the germinal vesicle (GV), chromosome condensation, spindle formation, and separation of homologous chromosomes, extrusion of the first polar body, and arrest of the meiotic process at metaphase II.

To increase the number of mature oocytes, standard in vitro fertilisation (IVF) protocols require ovarian stimulation with high dosage of gonadotrophins, with the accompanying risk of developing an ovarian hyperstimulation syndrome (OHSS). Infertile women with polycystic ovaries (PCO) or polycystic ovary syndrome (PCOS) are particularly sensitive to stimulation with exogenous gonadotrophins, and are at increased risk of developing OHSS. In vitro maturation (IVM) of immature oocytes, retrieved from unstimulated ovaries, would overcome this risk. Since reports of the first successful births,[1–3] more than 1000 children have been born from IVM procedures, particularly in patients with PCOS.[4 5] However, after IVM, the pregnancy rates remained lower than after regular IVF.[6 7]

Recent reports have associated artificial reproductive techniques (ARTs) with increased incidence of imprinting disorders such as Beckwith–Wiedemann syndrome (BWS),[8–11] Angelman syndrome,[12 13] and retinoblastoma[14] in children conceived with the use of these procedures. Genomic imprinting is an epigenetic form of gene regulation that leads to a parent-of-origin monoallelic expression of certain genes. Their allele specific expression is mainly regulated by the differential methylation of cytosine residues, within CpG dinucleotides, at differentially methylated regions (DMR).[15] Imprints are erased in primordial germ cells early in fetal life, and re-established according to the sex during gametogenesis. In the mouse, de novo methylation of CpG sites in DMRs occurs during the oocyte growth phase, in a size dependent manner, and with an independent timing for each imprinted gene.[16 17] Therefore, the use of gonadotrophin in classical IVF procedures may cause the release of immature oocytes with incomplete or labile imprints. Similarly, the establishment of maternal imprints might be affected by in vitro maturation of oocytes since embryo culture has been demonstrated to influence the methylation status and the expression of murine H19/Igf2[18–20] and Kcnq1ot1,[19] and human oocytes matured in vitro harboured methylation at H19 DMR1.[21]

KCNQ1OT1 lies on human chromosome 11p15.5, a region organised into two domains controlled by two distinct imprinting control regions, DMR1 and KvDMR1. DMR1 is methylated on the paternal allele and regulates the expression of H19/IGF2. KvDMR1 is located in the promoter of the non-coding *KCNQ1OT1* gene and is maternally methylated. KCNQ1OT1 is paternally expressed and is thought to regulate negatively the expression of several maternally expressed genes.[22] Loss of methylation (LOM) on the maternal allele has been observed in 40–50% of all sporadic BWS cases[23–25] and in the majority of BWS patients conceived through ART.[10 11] To evaluate the impact of either ovarian stimulation with gonadotrophin or in vitro maturation on the establishment and/or maintenance of the maternal imprint, we analysed the methylation profile of the imprinting centre KvDMR1 in human oocytes at different phases of their maturation.

MATERIAL AND METHODS

Source of human oocytes and embryos

The oocytes were donated for research by patients of Laboratoire de Biologie de la Reproduction at

Edouart Hérriot Hospital, after they had given their consent. A total of 167 oocytes were collected from 52 patients included in an intracytoplasmic sperm injection (ICSI) protocol and undergoing standard long term stimulation with follicle stimulating hormone (FSH) and human chorionic gonadotrophin (HCG). Most of the women do not have any known infertility problems, but 11 suffered from PCO, three from dysovulation, four had endometriosis, and one autoimmune lupus. The oocytes collected for research were immature (either at germinal vesicle (GV) or at metaphase I (MI)), or at metaphase II (MII) stages. Immature oocytes were either directly saved for methylation analysis or matured in vitro during a 28 h period[26] before analysis. A total of 70 oocytes were collected from patients with PCO during natural cycles, following a 28 h culture in vitro. The in vitro medium was from Medicult R (Mollehaven, Jyllingy, Denmark) supplemented with FSH, HCG, and 10% serum from the patient. The oocytes were denuded of cumulus cells by repeated pipetting in a hyaluronidase solution (150 units, type VII; Sigma, La Chapelle sur Erdre, France). Zona pellucida and any remaining somatic cells attached were removed by digestion with proteinase K (9 units/ml). One to six oocytes at the same stage of maturation and from the same cycle were pooled.

DNA methylation analysis

The methylation profile of KvDMR1 was determined by bisulfite mutagenesis and sequencing as previously described.[21] We analysed a total of 22 CpG sites in a 260 bp fragment (66531–66801 bp, U90095) following nested polymerase chain reaction (PCR). To set up the bisulfite sequencing technique at the single cell level, particular care was taken in the choice of primers for nested PCR: selected external primers should typically give a good signal on agarose gel, starting with 1 ng of bisulfite treated genomic DNA, and internal primers should give a signal amplifying the first PCR product obtained with 10 pg of genomic DNA. Primers specific for bisulfite converted DNA: external forward, 5′-TGTTTTTGTAGTTTATATGG AAGGGTTAA-3′; external reverse, 5′-CTCACCCCTAAAAA CTTAAAACCTC-3′; internal forward, 5′-GTTAGGGAAGTT TTAGGGTGTGAAT-3′; internal reverse, 5′-AAACATACC AAACCACCCACCTAACAAA-3′.

The PCR products were cloned into pGEM-T (Promega) and sequenced. Four to six clones were sequenced for each product. Because of the limiting starting material and the destruction of the DNA that is inherent to bisulfite treatment, identical sequences from separated PCRs are certain to represent distinct chromosomes, but identical sequences from one product are counted only once, as previously discussed[27] The frequency of the two methylation states found in different oocyte populations were analysed using distribution free tests.[28]

Approximately 100 ng of the same PCR product used for cloning and sequencing was digested with TaiI (ACGT/) and PauI (G/CGCGC).

RESULTS

Analysis of the methylation status of KvDMR1 in oocytes from stimulated cycles

To determine whether the maternal methylation profile of imprinted genes was established in fully grown GV oocytes and whether the 28 h culture period necessary to mature GV oocytes in vitro could influence the establishment/maintenance of the imprint, we examined the methylation pattern of the KvDMR1 region in oocytes at GV, MI or MII stages, retrieved from stimulated cycles. A total of 167 oocytes from 52 stimulated cycles were included in this study. Eleven patients had PCO, three suffered from dysovulation, two from endometriosis, one had an autoimmune lupus, and the other 33 exhibited normal fertility parameters. Forty oocytes at the GV stage from 12 patients (mean (SD) age 31.36 (1.36) years), 27 oocytes at the MI stage from 12 patients (30.25 (1.38) years), and 19 oocytes at the MII stage from three patients (31.11 (1.66) years) were collected for analysis immediately after retrieval. The methylation status of a 22 CpG site segment within KvDMR1 (fig 1A) was determined using bisulfite sequencing analysis adapted to the single cell level. KvDMR1 is unmethylated in spermatozoa, as expected, and both methylated and unmethylated alleles were equally obtained from either lymphocytes or cumulus cells (fig 1B), which exclude any bias in the PCR amplification between methylated and unmethylated strands. Optimisation of the bisulfite sequencing protocol at the few-cell level allow us to analyse oocytes (1–3 oocytes per PCR amplification cycle) from each

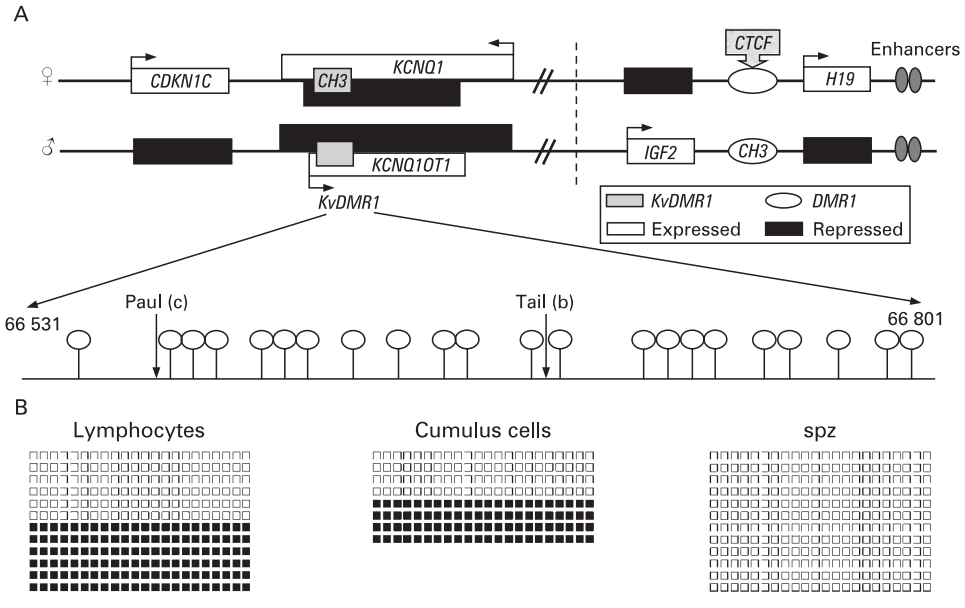

Figure 1 (A) Map of the 11p15 imprinted region comprising KvDMR1. The enlargement below the map shows the position of the differentially methylated CpGs analysed as well as PauI and TaiI sites. Base numbering is according to GenBank accession number U90095. (B) Bisulfite sequencing analysis of KvDMR1 in lymphocytes, cumulus cells and spermatozoa. Each line represents a single allele. A black square indicates a methylated CpG, an open square denotes an unmethylated CpG.

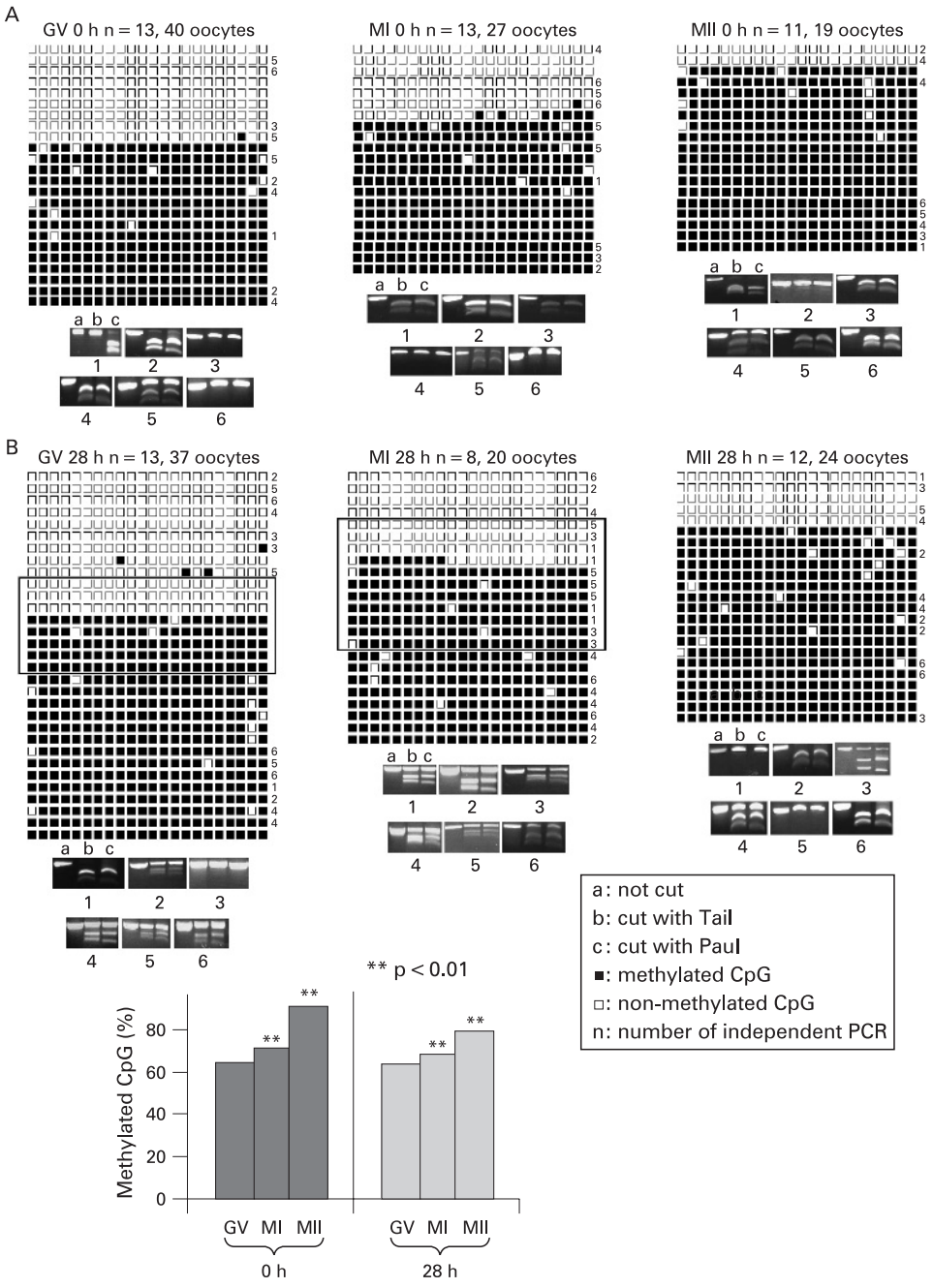

Figure 2 Methylation status of KvDMR1 in oocytes from stimulated cycles. Bisulfite sequencing analysis was performed on oocytes at different stages of their development—germinal vesicle (GV), metaphase I (MI) or metaphase II (MII): (A) directly following collection (0 h) or (B) following in vitro culture for 28 h. The number of independent experiments (n =) and the total number of oocytes analysed are indicated for each stage. Each row of squares represents a single allele. A black square indicates a methylated CpG, an open square denotes an unmethylated CpG. The numbers on the right correspond to the polymerase chain reaction (PCR) products shown below each diagram, uncut (a) or following digestion with TaiI (b) or PauI (c). Dynamics of methylation change during oocyte maturation is summarised in the diagrams below the figure. In panel B, encircled alleles are from patients with polycystic ovaries; they correspond to four independent PCRs performed on 15 oocytes' DNA for GV and three independent PCRs performed on eight oocytes' DNA for MI. For each sample, the methylation data were analysed by computing the percentage of methylated CpGs out of the total number of CpGs analysed and subjected to statistical analysis using distribution free tests.[28]

patient individually. The efficiency of PCR amplification was 63.8% (99 positive PCR amplifications/155). Fully grown GV oocytes (120–150 μm in humans) exhibited heterogeneous methylated profiles, with 62.5% of the alleles being methylated, corresponding to 60.4% of the total CpG sites analysed (319/528) (fig 2A). The percentage of methylated alleles in MI oocytes was more elevated than in GV oocytes (66.6%/62.5%, corresponding to 308 methylated CpG sites/462 analysed CpGs; $p<0.01$) (table 1). In contrast, most alleles were methylated in MII oocytes (89.5%, corresponding to 364 methylated CpG sites/418 analysed CpGs), although two alleles from two different patients remained hypomethylated at this last stage of oocyte differentiation before fertilisation. Therefore, de novo methylation of KvDMR1 occurred with meiosis II progression.

To evaluate the effect of in vitro culture on the ontogeny of the methylation at KvDMR1, GV oocytes retrieved from patients treated with gonadotrophins for ICSI were cultured in an in vitro maturing medium for 28 h.[26] At the end of the culture period, the oocytes either stayed at the GV stage of their first meiotic division (37 oocytes from 10 patients, four of which had PCO; 32.38 (0.87) years) or remained arrested at the MI stage (20 oocytes from eight patients, three of which had PCO; 28.75 (1.29) years) or undergo their second meiotic division up to the normal block before fertilisation at MII (24 oocytes from 12 patients, 32.08 (1.23) years). Following this 28 h culture period, arrested GV or MI oocytes exhibited a heterogeneous methylated profile similar to oocytes at the same stage which were not subjected to in vitro culture (61.2%/62.5% methylated alleles, corresponding to 408 methylated CpG sites/682 analysed CpGs compared to 319/528, $p>0.05$ for GV and 65.2%/66.6% methylated alleles, corresponding to 327 methylated CpG sites/506 analysed CpGs compared to 308/462, $p>0.05$ for MI

oocytes) (fig 2B) (table 1). De novo methylation of KvDMR1 also occurred in MII oocytes matured in vitro, but the methylation level was lower in this case (78.3%/89.5% methylated alleles, corresponding to 380 methylated CpG sites/506 analysed CpGs compared to 364/418; $p<0.01$). It is of note that the methylation level of GV and MI oocytes from stimulated PCO patients (encircled alleles in fig 2B) matched that of the total population of in vitro cultured GV and MI oocytes from stimulated cycles (62.5%/61.2% methylated alleles, corresponding to 107 methylated CpG sites/176 analysed CpGs compared with 408/682 for GV $p\geq0.9$ and 63.6%/65.2% methylated alleles, corresponding to 157 methylated CpG sites/242 analysed CpGs compared with 327/506 for MI, $p\geq0.9$).

Because the bisulfite treatment was done on a limited number of copies, restriction analysis with enzymes that cleave only the methylated template was performed to verify that the sequencing results reflect the overall methylation profile of KvDMR1 in the oocytes analysed. Digestion with PauI (G/CGCGC) and TaiI (ACGT/) were carried out on every PCR product before cloning. As can be seen in the examples shown under the methylation profiles for each group of oocytes (fig 2), if the sequencing shows only unmethylated profiles, the PCR product was not cut by both enzymes and appears on agarose gel as a band of 291 bp; methylated templates gave fully digested bands, PauI generates two fragments of 195 and 95 bp, TaiI generates two fragments of 161 and 129 bp; and digestion of heterogenous methylated templates revealed a mixture of uncut and cut products. With the reservation that this gives information on four CpG sites only, the results of restriction enzyme analysis are analogous to those obtained from sequencing, excluding any bias during cloning.

Analysis of the methylation status of KvDMR1 in oocytes from unstimulated cycles

To determine the possible interference of gonadotrophin treatments with the ontogeny of methylation at KvDMR1, we examined the methylation pattern of 70 oocytes that were retrieved from 16 natural cycles in women with PCOs and underwent in vitro maturation. MII oocytes were not available for analysis because they were subjected to sperm injection in the course of the parental project of the couple. Oocytes remaining arrested at GV (36 oocytes from nine patients, 29.5 (0.85) years) or MI stages (34 oocytes from eight patients, 30.5 (0.97) years) were de facto excluded from the parental project and were included in the study. Following a 28 h culture period, the percentage of methylated alleles was significantly increased in MI oocytes compared to GV oocytes (70.3%/67.8%, corresponding to 413 methylated CpG sites/594 analysed CpGs compared to 414/616; $p<0.01$) and KvDMR1 was significantly more methylated in GV oocytes retrieved from natural cycles than in GV oocytes from stimulated patients (67.8%/61.2% methylated alleles, corresponding to 414 methylated CpG sites/616 analysed CpGs compared to 408/682; $p<0.001$) (fig 3) (table 1). This is also true when only GV from PCOs stimulated patients are considered (67.8%/62.5% methylated alleles, corresponding to 107 methylated CpG sites/176 analysed CpGs compared to 414/616; $p<0.01$) (fig 2B and fig 3). Likewise, KvDMR1 is more methylated in MI oocytes from natural cycles than from stimulated cycles in both the general population (70.3%/65.2% methylated alleles, corresponding to 413 methylated CpG sites/594 analysed CpGs compared to 327/506; $p<0.01$) and the PCO population (70.3%/63.6% methylated alleles, corresponding to 413 methylated CpG sites/594 analysed CpGs compared to 157/242; $p<0.01$).

As previously explained, digestions with PauI and TaiI were carried out on each PCR product to be cloned and sequenced, and results of restriction enzyme analysis are comparable to those obtained from sequencing (examples are given under the methylation profiles of each group of oocytes in fig 3).

Table 1 Percentage of methylated alleles in germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) oocytes in the different experimental situations

	Stimulated cycles			Unstimulated cycles
	Matured in vivo	Matured in vitro	PCO/matured in vitro	PCO/matured in vitro
GV	62.5	61.2	62.5	67.8
MI	66.6	65.2	63.6	70.3
MII	89.5	78.3	–	–

PCO, polycystic ovaries.

DISCUSSION

A series of recent observations has indicated an increased risk of conceiving a child with an imprinting defect leading to several diseases and syndromes in couples who procreated using ART. In particular, the risk of BWS was up to nine times greater in the ART population than in the general population.[29] BWS is characterised by pre- and/or postnatal overgrowth, a series of abnormalities, and a predisposition to embryonal tumours (MIM 130650). ART related BWS is specifically associated with loss of maternal methylation at the KvDMR1 imprinting control region. In all the published cases of ART related BWS, no common factor could be identified—the causes of infertility, the stimulatory protocol, the culture media used, the biological technique, as well as the timing of embryo transfer varied.[10 11 29] The only general feature is the use of ovarian stimulation protocol and, in most of the cases, of in vitro culture. IVM of immature oocytes retrieved from unstimulated ovaries may overcome a possible disturbing effect of hormonal stimulation on methylation of the maternal genome, but will not eliminate the potential risk associated with the in vitro culture of the oocytes. The availability of immature oocytes at the GV or MI stages from stimulated and unstimulated cycles gave us an opportunity to appreciate the impact of each factor on the methylation status of KvDMR1.

We first show that the hypermethylated pattern characteristic of the maternal allele of KvDMR1 in somatic cells is only established in two thirds of total alleles analysed in fully grown GV oocytes from stimulated cycles. These results are apparently divergent from those of Geuns *et al*[30] who showed a fully methylated pattern in GV oocytes. The discrepancy between both studies could be due to the small number of independent alleles randomly sequenced in Geuns *et al*[30] (seven for GV and seven for MI in Geuns *et al*[30] compared to 55 and 44, respectively, in this study). More likely, since the region analysed by this group is situated 1239 bp downstream from the region in the present study, it may reflect a dichotomy between two regions within the DMR whose imprinting schedule would be dissociated. The fact that methylation would not occur by linear spreading along the chromosome has been previously documented for the H19-Igf2 locus: a hierarchy in the methylation process has been observed during early post-implantation development, the methylation of DMR1 preceding that of DMR2 in the kidney.[31]

In mice, KvDMR1 is entirely methylated in early antral follicles, before meiosis resumption.[17] This contrast between

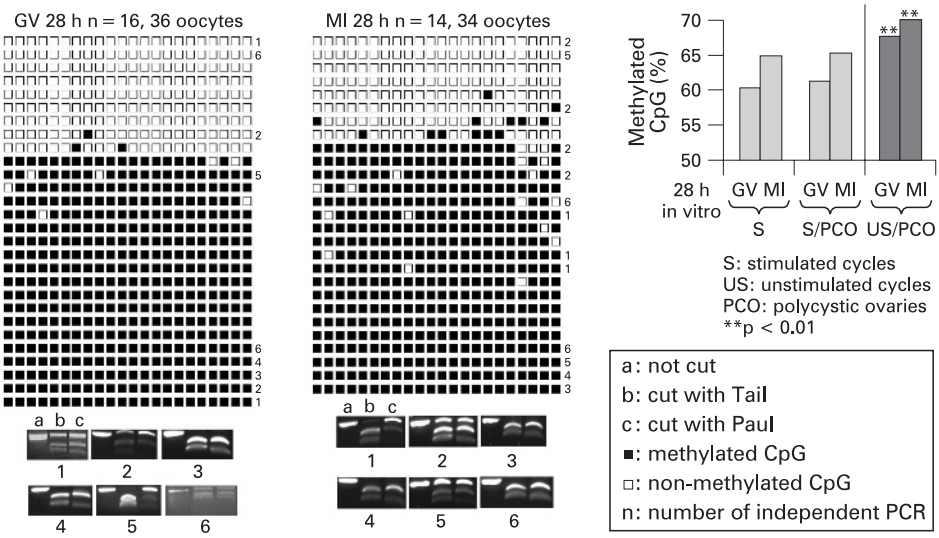

Figure 3 Methylation status of KvDMR1 in oocytes obtained from natural cycles in patients with polycystic ovaries (PCO). Only germinal vesicle (GV) and metaphase I (MI) oocytes could be analysed. Details are described in fig 2. Comparisons with results obtained from stimulated cycles in all patients or in PCO patients only are summarised in the diagram. For each sample, the methylation data were analysed by computing the percentage of methylated CpGs out of the total number of CpGs analysed and subjected to statistical analysis using distribution free tests.[28]

mice and humans emphasises the need for studies on humans. De novo methylation progresses through meiosis I and II, and almost all matured MII oocytes were fully methylated at KvDMR1. As a few DNA copies remained unmethylated at the MII stage, we cannot exclude the possibility that maternal methylation of KvDMR1 could occur at later stages. Some of the oocytes that were immature at the time of collection succeeded in completing maturation in vitro within 28 h. Since the imprint was not thoroughly established in fully grown GV oocytes, we wondered whether the culture media could influence the de novo methylation of KvDMR1. Following in vitro culture, immature oocytes from stimulated cycles that remained at the GV stage or progressed to MI exhibited the same degree of methylation than oocytes matured in vivo, but MII oocytes showed a significant deficit in methylation. Several hypotheses may account for this observation. It could be attributed to the shorter time given to GV oocytes to mature in vitro compared to the in vivo maturing period (28 h/36–40 h), which does not allow the methylation process to go to completion. On the other hand, prolonged culture of MII oocytes has been shown to induce demethylation of Peg1/Mest DMR in the mouse.[32]

In the natural human menstrual cycle about 15 follicles are recruited for growth. After some 14 days, only one or two of these follicles reach maturation and are ovulated. In standard ART, the large dose of gonadotrophins used to rescue the growth of small follicles could cause the premature release of oocytes that are at an earlier developmental stage. These oocytes have escaped selection and may not be able to overtake their delay in acquisition of the imprint and therefore exhibit a hypomethylated pattern at MII. The hypothesis that gametic imprints are sensitive to in vitro conditions is supported by previous work: a gain of methylation has been described at H19/Igf2 DMR following in vitro culture of preimplantation embryos, couple with altered development,[18 33] and a loss of methylation at the Igf2R and Peg1/Mest loci in fully grown GV oocytes produced by in vitro development of preantral follicles.[34] Thus, we cannot exclude the possibility that the impaired de novo methylation of KvDMR1 observed in stimulated oocytes matured in vitro may be due in part to the composition of the culture media that does not exactly match the in vivo environment.

Oocytes obtained from unstimulated cycles have been subjected in vivo to a surge of circulating FSH that stimulates follicular recruitment, but they are collected before dominance and atresia of the subordinate follicles. They represent a valuable control to evaluate a potentially negative impact of superovulatory gonadotrophin treatments on imprint establishment. At the end of the in vitro maturing process, MII oocytes were never available because they were selected for the ICSI procedure. We observed in both GV and MI oocytes from natural cycles a significant increase in methylation as compared to oocytes at the same stage of maturation retrieved from stimulated cycles. This is particularly true when oocytes in both cycles were exclusively from POC patients; this means that the higher methylated state observed in natural cycles is not related to the aetiology of infertility—that is, PCOs in this case. One may fear that in PCO patients the oocytes recovered from natural cycles might include degenerating and atretic follicles which we did not observe. The high methylated state observed in GV and MI oocytes, and the increase of de novo methylation observed between both stages, are in favour of full imprint establishment by MII stage, though MII in vitro matured oocytes from natural cycles are not available, so it cannot be verified. The deficit of methylation in oocytes from stimulated cycles is likely to reflect the recruitment of small follicles that are at an earlier stage of development. Those oocytes may not be able to acquire full imprint by MII, even though they appeared mature in other respects and could be fertilised.

Our study on a large cohort of human oocytes demonstrated that imprint establishment proceeds during in vitro maturation, which is of particular importance for the thousands of children born from IVM oocytes. We also showed that the high concentration of gonadotrophins provided to antral follicles before recruitment to terminal growth led to the selection of some oocytes that failed to acquire full imprint at KvDMR1, particularly when they were matured in vitro, very likely because they were at an earlier stage of development. Our results are in agreement with other studies on epigenetic disorders in children born from ART that could not establish a correlation between a particular technique and the imprinting defect observed, with the exception of the hormonal treatment.

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Competing interests: None declared.

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CORRESPONDENCE

No reason yet to change diagnostic criteria for Noonan, Costello and cardio-facio-cutaneous syndromes

The clinicogenetic relationship between Noonan syndrome (NS) and Noonan-like syndromes (Costello syndrome (CS), cardio-facio-cutaneous (CFC syndrome) and the disruption of the RAS-ERK pathway makes for a fascinating story that has developed over the past decade, and is still developing. The discovery that the genes causing these syndromes encode proteins that converge on the same metabolic pathway facilitated understanding of the similarities that group these conditions within one family of syndromes.[1] Nevertheless, their nosological classification continues to be a challenge. There is general agreement that NS is caused by mutations of *PTPN11* (most cases), *SOS1* or *RAF1*, that CFC syndrome is caused by mutations of *BRAF* (most cases), *MEK1* or *MEK2*, and that CS is caused by mutations of *HRAS*. Cases due to *KRAS* mutations are of uncertain nosological classification, and there are some borderline cases for which the clinical diagnosis will be uncertain even for the best experts in the field.

The recent article by Nyström *et al*[2] represents another attempt at bringing some order within this complex matter. However, we think that some of the conclusions drawn by the authors are unwarranted. The main focus of this letter is on patients 3 and 8 of that study, who are unusual because, according to the authors, the former has CFC syndrome caused by a mutation in *SOS1*, and the latter has NS, caused by a mutation in *BRAF*.[2] The inescapable conclusion is a further expansion of the genetic heterogeneity of both syndromes. However, it is an arguable conclusion. Although we can judge only on the basis of a very sparse clinical description in the table and small photographs in the first figure, our impression is that patient 3 is a typical case of *SOS1* NS (no mental delay, no curly hair, dry skin, missing eyebrows), and patient 8 is an ordinary case of CFC syndrome due to *BRAF* mutation (mental delay, curly hair, other typical features). Nyström *et al*[2] state that their clinical diagnoses were based on criteria established by Roberts *et al*,[3] but they had to make exceptions (arbitrarily) on at least two criteria: mental delay and curly hair. Moreover, they did not consider that Roberts *et al*[3] published their criteria before the discovery of *SOS1*,[4] which established the association between NS and pronounced skin involvement. Moreover, it should be pointed out that Nyström *et al*[2] based their diagnoses of CFC or NS solely on presence or absence of ectodermal signs that by themselves do not capture the complexity of these phenotypes, resulting occasionally in misdiagnosis.

Based on these considerations, we see no reason, at the moment, to change the generally held view that NS can be caused by mutations in three different genes (*PTPN11*, *SOS1*, *RAF1*), CFC syndrome by mutations in another three genes (*BRAF*, *MEK1*, *MEK2*), and CS by *HRAS* mutations, with *KRAS* mutations causing rather atypical cases that are difficult to classify. As Nyström *et al*[2] did not discuss CS in their report, it is surprising and difficult to understand why they indicate that this condition can be caused not only by *HRAS* mutations, but also by mutations in *KRAS*, *BRAF* and *MEK1* (their third figure). There is general agreement that the diagnosis of CS should be restricted to those cases that carry a *HRAS* mutation,[5] which implies increased risk for cancer and consequent application of a strict surveillance protocol. Again, there is no reason, at the moment, to change this stance.

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CORRECTIONS

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Lugtenberg D, de Brouwer APM, Kleefstra T, Oudakker AR, Frints SGM, Schrander-Stumpel CTRM, Fryns JP, Jensen LR, Chelly J, Moraine C, Turner G, Veltman JA, Hamel BCJ, de Vries BBA, van Bokhoven H, Yntema HG. Chromosomal copy number changes in patients with non-syndromic X linked mental retardation detected by array CGH. *J Med Genet* 2006;**43**:362–70.

There was an error in an article published in the April 2006 issue of the journal. The authors reported possible duplication of copy number polymorphisms flanking the *MECP2* region. These duplications were observed by multiplex ligation-dependent probe amplification (MLPA) in one patient and two controls (figure 2). Recent further investigations of these regions have shown that these duplications were false observations. Newly synthesised MLPA probes and better quality DNA samples showed no extra DNA copies in the region. From reanalysis of the previously obtained MLPA data, the authors conclude that the fluorescence intensity signals were outside the normal range in which the data can be correctly interpreted (100–1000) for current quality-control thresholds (>10000). The authors regret this incorrect interpretation of the data.

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There was an error in an author's name in an article published in the September issue of the journal (Khoureiry R, Ibala-Rhomdane A, Méry L, Blachère T, Guérin J-F, Lornage J, Lefèvre A. Dynamic CpG methylation of the *KCNQ1OT1* gene during maturation of human oocytes. *J Med Genet* 2008; **45**:583–8). The name of the first author is R Khoueiry.