Imprinting: RNA expression for homocysteine recycling in the human oocyte

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Objective: To investigate whether homocysteine, a well known inhibitor of methylation, which is produced after imprinting and other methylation processes, can be recycled to methionine in the oocyte, at least until the stage of maternal to zygotic transition (i.e., four- to eight-cell stage); before this stage, most of the biochemical processes are carried out with the use of maternal stores of protein and mRNA.

Design: A first approach using microarrays and then reverse-transcription polymerase chain reaction (RT-PCR) for methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase [MTR]), betaine-homocysteine methyltransferase (BHMT), and cystathionine-beta synthase (CBS).

Setting: Two private hospitals.

Patient(s): Patients involved in IVF/ICSI procedures.

Intervention(s): Germinal vesicle oocytes collected at the time of oocyte retrieval, RNA extraction amplification, RT-PCR, microarrays.

Main Outcome Measure(s): mRNA expression of all the enzymes involved in the chain of methylation and recycling of homocysteine to methionine.

Result(s): All of the enzymes required for methylation are present in the oocyte. Homocysteine can be recycled with BHMT and MTR.

Conclusion(s): The human oocyte is able to regulate its Hcy level via remethylation using MTR and BHMT but not CBS. This aspect is important, because recent studies have shown that controlled ovarian hyperstimulation affects the homocysteine concentration in follicular fluid. This may regulate, at least in part, the risk of imprinting problems during IVF procedures. (Fertil Steril® 2009;■:■–■. ©2009 by American Society for Reproductive Medicine.)

Key Words: Imprinting, human oocyte, homocysteine, methionine, methylation, mRNA

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The importance of imprinting in establishing the necessary complementarities between the maternal and the paternal genomes to ensure normal embryonic development is now common knowledge. Imprinting is an epigenetic process that involves a type of "dialogue" that takes place during embryonic development as early as the pronuclear stage. Many human diseases may have significant epigenetic components. An epigenetic process plays an important role in many cancers that appear late in life (1, 2) and may have its roots in epigenetic inheritance or epigenetic variation established in early development. One of the very early events that is regulated epigenetically is the methylation of DNA and histones, catalyzed by DNA methyltransferase with S-adenosyl-methionine (SAM) as a methyl donor. SAM and other methylation agents (group B vitamins) are the critical epigenetic regulators that can affect DNA and histone methylation and imprinting (3–5), as well as histone H3 methylation (4, 6). Imprinting is also a regulator of implantation (7). S-Adenosyl-homocysteine (SAH) is formed as a product of methylation, and then homocysteine (Hcy) is released. High concentration of Hcy in the oocyte (8) and embryo environment is deleterious, because it induces efflux of methionine (Met) (9), which then has a negative impact on the methylation process, directly or indirectly. Insufficient remethylation of Hcy to Met leads to decreased SAM production and lowers the SAM/SAH ratio, leading to DNA hypomethylation. Insufficient availability of SAM then impairs all methylation reactions: Anomalies in methylation patterns lead to biallelic expression or repression of the imprinted genes, and this then induces malformation (10).

Recent observations have highlighted the possibility that assisted reproductive technologies (ART) could increase the risk of imprinting problems for the conceptus (5, 11) and may be associated with imprinting-associated syndromes, such as Beckwith-Wiedeman and Praeder-Willy syndromes. CpG methylation in the PWS-1C site, linked to Prader-Willy syndrome, occurs after fertilization in humans (12). In contrast, in the mouse the corresponding site is methylated during oogenesis. Imprinting defects may in particular be related to increased maternal age and controlled ovarian hyperstimulation (13) as well as poor sperm quality (14, 15). The requirement for Met is also significant because of the role of methionyl-tRNA as the initiator of protein syntheses. SAM also leads to synthesis of spermine and spermidine, both actively involved in regulation of embryonic DNA synthesis and growth. Homocysteine can be removed via a trans-sulfuration pathway which leads to the production

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of cystathionine and cysteine in bovine and mouse embryos (16, 17). However, homocysteine can also be remethylated into methionine, with the help of methyl donors such as 5-methyltetrahydrofolate (5-methyl-THF) and betaine. Two major enzymes are involved in these pathways: methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase [MTR]) and betaine-homocysteine methyltransferase (BHMT). In the present paper, we focus our attention on the expression of the mRNAs coding for these enzymes in human germinal vesicle (GV) oocytes for ethical reasons as well as because the first steps of preimplantation embryo development up to the stage of maternal to zygotic transition (i.e., four- to eight-cell stage) are carried out with the use of maternal stores of protein and mRNA (18, 19).

MATERIALS AND METHODS

French bioethics laws prohibit research on oocytes and embryos that can be used by patients undergoing IVF treatments. Therefore, all of the experiments were performed on GV oocytes after careful denudation of all oocytes retrieved in preparation for intracytoplasmic sperm injection. According to governmental regulation (Agence de Biomedecine) a written consent was signed by the patients, although this was not strictly necessary, because GV oocytes are normally discarded. The oocytes were collected from two different IVF units, both using a current protocol of semilong GnRH analogue treatment followed by stimulation with FSH or hMG. The oocytes were pooled in groups (two pools of 30, two pools of 40, one of 60, and one of 70) and kept frozen in liquid nitrogen.

The pooled oocytes (270 in total) were divided into nine samples for an average of 30 oocytes per sample: six samples were used for microarray assays, and the other three were used for specific gene expression analysis by PCR and gel analysis.

Microarrays

The protocol used for the human oocytes was as previously published (20). The analyses were performed with microarrays, using the Affymetrix HG U-133 plus 2.0 chips. Amplification was performed via two cycles of in vitro transcription.

Data were normalized using the algorithm MAS5 to generate a signal for each of the 54,613 transcripts of the chips (Probe set ID) that was proportional to the level of expression. A call signal points out and specifies if the gene is expressed at a level above the background. The expression level is given as high, medium, or low according to the signal. Housekeeping and spike controls were used for each determination. The methionine pathways linked to methylation and recycling of Hcy are summarized in Figure 1.

Gene-Specific RT-PCR

RNA extraction From three samples containing 30 and 40 oocytes, after zona pellucida digestion with pronase, the total RNA was extracted using Trisol reagent (Invitrogen, Cergy Pontoise, France) following the manufacturer's recommendations. Briefly, after oocyte lysis and phase separation, the total RNA was purified by a binding step and RNA spin cartridge. To collect the purified RNA, two additional steps of washing and elution were applied. Finally, the purified RNA was resuspended in 30–40 μL RNase-free water and stored at -80°C.

Reverse-transcription polymerase chain reaction (RT-PCR) To produce total or specific cDNA products, 250 ng extracted RNA was reverse transcribed either in total by oligo-dT primers or by gene-specific primers using one-step reverse transcription kit (Qiagen, Courtaboeuf, France) and following the manufacturer's recommendations.

To confirm the positive expression of BHMT and MTR by PCR and gel analysis, we designed specific couples of primers for each gene. From the three samples after total RNA extraction, we performed total reverse transcription and specific gene transcription for both genes. For the internal control, an Amplimers set of primers of beta-actin gene was used (Clontech, St. Germain en Laye, France).

For BHMT, the following forward primer was designed: GGATTCATCGATCTCCAGA; completed by the reverse sequence CAGCTGCCATGTTTTTCTGA. The PCR mix contained: 12.5 μL Multiplex Master Mix, 0.25 μL RT Mix, 5 μL BHMT and beta-actin primers (0.5 μmol/L each), and 250 ng RNA or cDNA. To obtain 261-bp and 752-bp products for BHMT and beta-actin, we applied the following PCR program: 30 minutes at 50°C followed by 15 minutes at 95°C and then 37 cycles of 30 seconds at 95°C, 30 seconds at 63°C, and finally 1 minute at 72°C.

For MTR gene, the designed left- and right-side primers were: GACGACATTCACCTGTACGC and AGAGAACACTCACTTCTTCA. The PCR mix contained: 12.5 μL Multiplex Master Mix, 0.25 μL RT Mix, 5 μL MTR and beta-actin primers (0.6 μmol/L) and 250 ng RNA or cDNA. To obtain 620-bp and 752-bp products for MTR and beta-actin coamplification, the following PCR program was applied: 30 minutes at 50°C, 15 minutes at 95°C, and then 42 cycles of 40 seconds at 95°C, 40 seconds at 63°C, and 1 minute at 72°C.

For cystathionine-beta synthase (CBS), we extracted the total RNA from peripheral blood cells in culture for karyotyping, as positive control. We used the previous procedure of one-step RT-PCR for the positive control and native RNA or cDNA from the oocyte pool. To obtain a PCR product of 194 bp for CBS gene on coamplification with beta-actin gene as internal control, we used forward CCTGAAGCCGCGCCCTCTGCAGATAAT and reverse GTCGCCGGGCTCTGGACTCGACCTACC primers. The PCR program was: 30 minutes at 50°C, 15 minutes at 95°C, and then 40 cycles of 45 seconds at 95°C, 15 seconds at 68°C, and 1 minute at 72°C.

All of the RT-PCR and PCR products were analyzed using 1.5% agarose gels and ethidium bromide staining.

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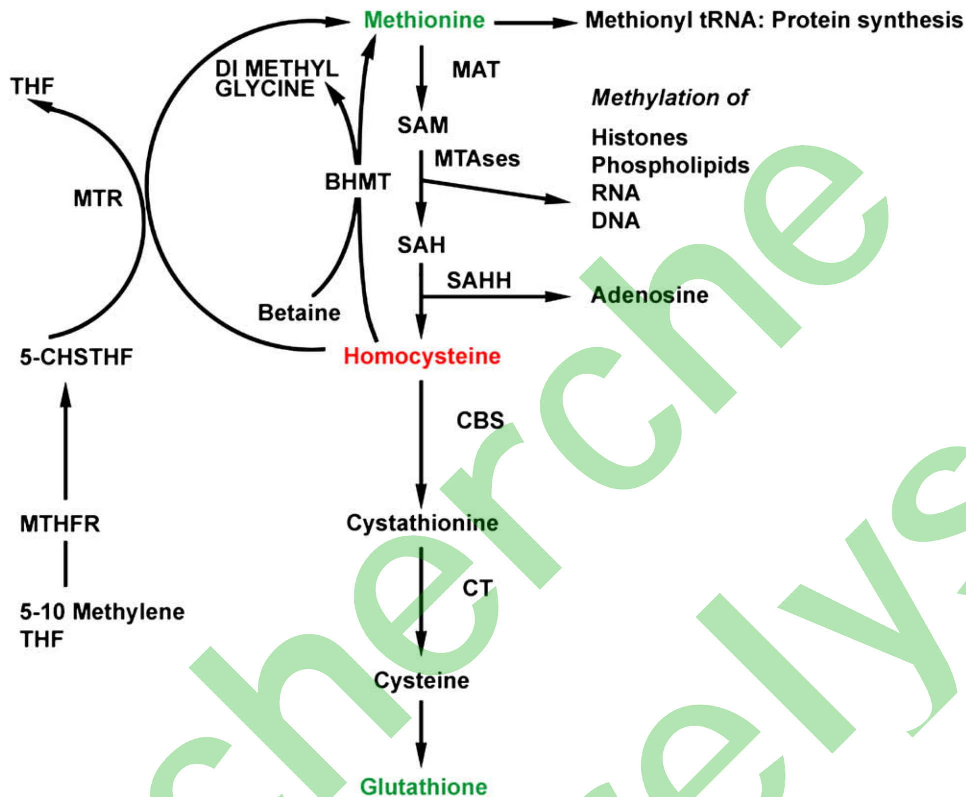
FIGURE 1

Recycling of homocysteine and methylation. BHMT = betaine-homocysteine methyltransferase; CBS = cystathionine-beta synthase; CT = cystathionase; MAT = methionine adenosyltransferase; MTases = various methylases; MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase); MTHFR = methylene tetrahydrofolate reductase; SAHH = S-adenosyl-homocysteine hydrolase; SAM = S-adenosyl methionine; THF = tetrahydrofolate.



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RESULTS

Using the microarray technology, 9,887, i.e., 18.1%, of the transcripts were expressed in the samples (Table 1, Figs. 2 and 3).

MTR-Folate Pathway

The signal for MTR was high: s = 2,589 (SD 1,600). The expression for 5,10-methylene tetrahydrofolate reductase (MTHFR) [s = 1,087 (SD 1,262)], methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) [s = 2,195 (SD 834)], and dihydrofolate reductase (DHFR) [s = 2,572 (SD 1,162)] had the same order of intensity. It was lower for methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 1 (MTHFD1): s = 903 (SD 927).

Gene Expression Related to BHMT Pathway

The signal for BHMT1 was always below the significance threshold [s = 46.4 (SD 29.9)], but it was positive in all the samples for BHMT2: s = 471 (SD 415).

CBS Pathway

We were unable to detect any expression signal for CBS; cystathionase (CTH) expression was found to be positive but variable (detected in only two-thirds of the samples): s = 343 (SD 365).

Expression of SAM/SAH Pathway

S-Adenosyl-methionine synthesis is active, as indicated by a high level of expression for methionine adenosyltransferase (MAT) II alpha [s = 3,625 (SD 2157)] and MAT II beta (s = 5,370 [SD 3,625]). S-Adenosyl-homocysteine hydrolase (SAHH) is also expressed [s = 1,405 (SD 986)]: Hcy is released from SAH after methylation originating from SAM.. The signal observed here for DNA methyl transferase 1 (Dnmt1) in the methylation process is also high and stable: s = 44,600 (SD 6,000).

Control

As a negative control, for quality of denudation (absence of cumulus cells), we found no signal for amphiregulin

(s = 25.1) and beta-cellulin (s = 15.2), which are normally released by the cumulus cells: All of the oocytes were correctly denuded.

Positive control: alpha-tubulin: s = 43,356 (SD 31,094); beta-actin: s = 10,343 (SD 5,863).

The results of PCR products are illustrated in Figures 2 and 3. The 620-bp band for MTR and the 261-bp band for BHMT2 can be detected in all of the samples. The CBS expression is negative in the oocyte and positive in peripheral blood (Fig. 4).

DISCUSSION

After fertilization, the first cleavage divisions are under maternal control, before the maternal to zygotic transition, and therefore analysis of oocyte stores yields an interesting picture of mechanisms in the early embryo. We previously observed that SAM is formed and Hcy is released in the human four-cell embryo, before genomic activation (9). Apart from certain specific regions, paternal DNA is globally passively demethylated, but female DNA undergoes de novo methylation in mammalian zygotes, including the human (12, 21, 22), essentially through Dnmt1 (23). The signal observed here for Dnmt1 was very high and stable [s = 44,600 (SD 6,000)]. In most mammalian cells, Hcy in excess has to be removed either by recycling into Met by remethylatio, or by transforming into cysteine by trans-sulfuration. This mechanism is even more necessary in the oocyte, where methylation must be regulated until the maternal to zygotic transition. Contrary to what has been described in mouse and bovine embryos with the use of a more sensitive method (^{35}S-radiolabeled methionine), CBS is not expressed in the human oocyte and the trans-sulfuration pathway does not seem to be active in removing Hcy. It is obvious that the biochemistry involving methyl donors is of major importance in the preimplantation embryo, from the time of fertilization and immediately after up to genomic activation. Silent paternal alleles of H19, Igf2, Grb10, and Grb7 are aberrantly

FIGURE 2

Methionine synthase (MTR) gene polymerase chain reaction (PCR) amplification products. Line A: ladder; line B: negative control PCR mix without primers, using native RNA; line C: PCR product from total cDNA after total RNA reverse transcription; line D: PCR product from specific RNA reverse transcription using gene-specific primers.

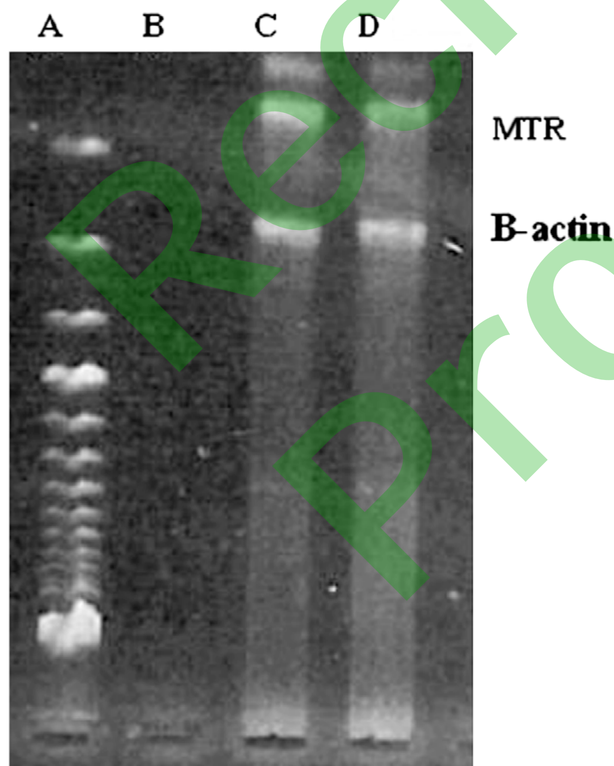

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FIGURE 3

Betaine-homocysteine methyltransferase 2 (BHMT) gene polymerase chain reaction (PCR) amplification products. Line A: PCR product from specific RNA reverse transcription using gene-specific primers; line B: PCR product from total cDNA after total RNA reverse transcription; line C: negative control PCR mix without enzyme; line D: ladder.

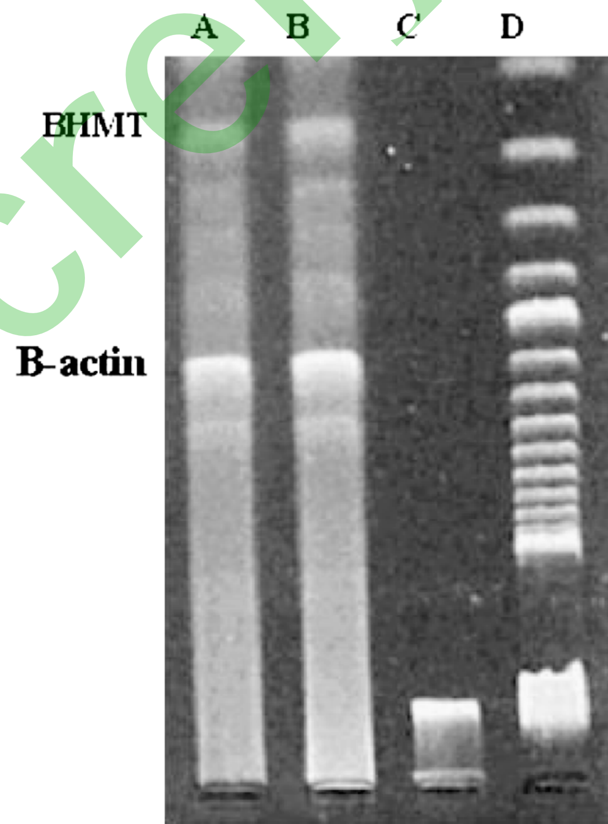

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FIGURE 4

Cystathionine-beta synthase (CBS) gene polymerase chain reaction (PCR) amplification products. Line A: positive control PCR product from specific RNA reverse transcription using gene-specific primers from peripheral blood RNA; line B: negative control reverse-transcription PCR mix without peripheral blood native RNA; line C: negative control using native peripheral blood RNA and without enzyme mix; lines D and E: negative result from direct native oocyte RNA; line F: negative result from oocyte cDNA; line G: ladder.

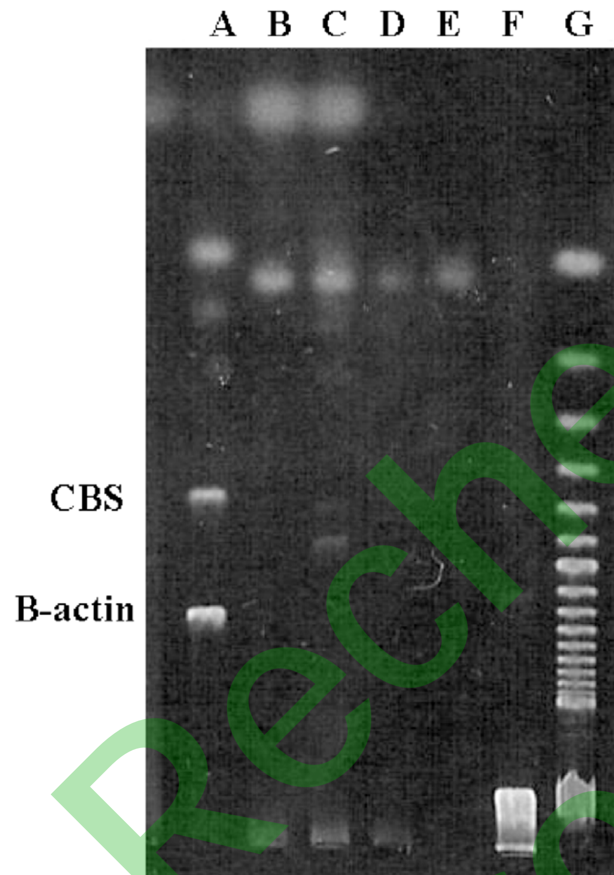

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TABLE 1

mRNA expression of the enzymes involved in homocysteine recycling (microarrays).

	Signal (SD)	Presence
MTR/folate pathway		
MTR	2,589 (1,600)	positive
MTHFR	1,087 (1,062)	positive
MTRR	2,195 (834)	positive
DHFR	2,572 (1,162)	positive
MTHFD1	903 (427)	positive
BHMT pathway		
BHMT1	46 (30)	negative
BHMT2	471 (415)	positive
CBS pathway		
CBS	52 (10)	negative
CT	343 (265)	positive[a]
SAM/SAH pathway		
MAT II alpha	3,625 (2,157)	positive
MAT II beta	5,370 (3,625)	positive
SAH hydrolase	1,405 (986)	positive
Negative control		
Amphiregulin	25 (24)	negative
beta-Cellulin	15 (15)	negative
Positive control		
alpha-Tubulin	43,356 (31,094)	positive
beta-Actin	10,343 (5,863)	positive

Note: BHMT = betaine-homocysteine methyltransferase; CBS = cystathionine-beta synthase; CT = cystathionase; DHFR = dihydrofolate reductase; MAT = methionine adenosyltransferase; MTHFD1 = methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 1; MTHFR = 5,10-methylene tetrahydrofolate reductase; MTR = 5-methyltetrahydrofolate-homocysteine transferase; MTRR = methyltetrahydrofolate-homocysteine methyltransferase reductase; SAH = S-adenosyl-homocysteine; SAHH = S-adenosyl-homocysteine hydrolase; SAM = S-adenosyl-methionine.

[a] Not expressed in all of the samples.

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expressed and hypomethylated in mouse embryos in Simplex media but not in media with amino acids (24). Microarray technology has also demonstrated that expression of genes is affected when embryos are cultured in media without amino acids (25). In fact, the balance between Met and the other amino acids is of major importance, because Met has a high affinity for carriers that regulate amino acid transport into the oocyte and early embryo (26). In ART, the Beckwith-Wiedemann syndrome appears to be age and methyl-donor dependent (13, 27). It is obvious that "ovarian hyperstimulation" disturbs the endogenous amino acid pool of the oocytes (including Met): The resulting methylation process is impaired if the amino acid content of the medium is not adapted.

Methionine is a so-called "essential amino acid." This class of amino acid is often omitted in most of the currently available culture media designed for cleavage-stage embryos (28), and this concept is highly questionable and even dangerous, especially in women over 35 years old, who are experiencing a progressive decline in biochemical regulatory functions in their oocytes. The question of intake of folic acid before and after ART technologies (2) and its presence in culture media has been addressed, yet the relation between maternal folic acid concentration and its content in the oocyte is totally obscure, although the presence of the reduced form of folic acid is mandatory in the embryo, especially for the synthesis of thymidine (29). Methionine must be added very early in

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embryo culture media: a side effect of Met lack could be a deficiency in endogenous embryo folic acid with a further negative impact on thymidine synthesis (29).

If we consider the biochemical pathways that are present, and take into account that at least a part of the mRNAs detected are translated, the human oocyte is able to keep its Hcy level in check via remethylation. This aspect is important, because recent studies have shown that controlled ovarian hyperstimulation affects the Hcy concentration in follicular fluid (30). This may serve to alleviate the inhibitory effect of Hcy on imprinting, but this can be modulated by maternal age and culture conditions (26). It should be mentioned here that the two methionine recycling pathways described here in the oocyte are also present in the testis (31).

The MTR pathway appears to be more important than the BHMT pathway, which is weakly expressed in only its "2" form. Finally, spermine and spermidine synthesis need (decarboxylated) SAM: These polyamines are embryotropic factors in some animal species, but this aspect is yet to be demonstrated in the human. However, using microarray technology we found an extremely high expression of SAM decarboxylase [s = 10,206 (SD 4,887)] in this study, which suggests that these compounds are being, actively synthesised in human GV oocytes and further on in the early preimplantation embryo.

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1 Imprinting: RNA expression for homocysteine recycling in the human oocyte

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Messenger RNA coding for some but not all of the homocysteine recycling pathways is present in the human oocyte.

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