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ASSISTED REPRODUCTION TECHNOLOGIES

Sequential (hFSH + recFSH) vs homogenous (hFSH or recFSH alone) stimulation: clinical and biochemical (cumulus cell gene expression) aspects

Timur Gurgan · Debbie Montjean · Aygul Demirol · Yves J. R. Menezo

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Abstract FSH is a key hormone in the regulation of follicular development. Together with the EGF network, these molecules mediate oocyte maturation and competence in preparation for the action of LH. FSH isoforms regulate distinct biological pathways and have specific effects on granulosa cell function and maturation of the ovarian follicle. Their dynamic interactions occur during the follicular cycle; short-living forms are predominant in the pre-ovulatory phase, whereas long-acting molecules characterize the luteal-follicular transition. Recombinant FSH (rFSH) molecules have a reduced number of isoforms and are less acidic, with a shorter half-life. We have investigated sequential stimulation, comparing hFSH + rFSH, vs. rFSH alone and hFSH alone for the entire stimulation phase. Sequential stimulation leads to an E2 per MII oocyte ratio that is much lower than is seen during treatment with the two drugs individually. Although there is a positive tendency in favor of the sequential treatment, there was no significant difference in pregnancy rates, even taking frozen embryos into consideration. The cumulus cell transcriptome varies considerably between the treatments, although with no clear significance. When comparing pregnant vs. non-pregnant patients, in general a decrease in mRNA expression can be observed in the pregnant patients, especially in expression of folic acid receptor 1 and ovostatin 2. This indicates that material has been transferred from CC to the oocyte. However, a common observation in the literature is that variations in the transcriptome of the cumulus cells are highly dependent upon the patient genotype; the potential for applying this strategy as a basis for selecting embryos is, at the very least, questionable.

Keywords hFSH · recFSH · Sequential stimulation · Cumulus cells · Transcriptome

Capsule A sequential stimulation protocol (hFSH + recFSH) has been compared to homogenous h FSH and rFSH protocol in three group of 30 patients. Clinical results and Cumulus cells (CC cells)gene expression has been analyzed. The sequential treatment leads to a E2 per MII oocyte ratio more physiological and much lower than the other treatment. It leads to better but not significant pregnancy rates, even taking frozen embryos into consideration. hFSH treatments appears to be rather associated with depletion, rather than over-expression of Poly(A) mRNAs: this means that the trophic transfer towards the oocyte has been completed. The CC cells mRNA content in the pregnant patients is depleted when compared to the non pregnant group. The usefulness of CC transcriptome analysis for selecting the best embryos is, at the very least, questionable, as some published statements are rather misleading.

T. Gurgan
Department of Ob&Gyn, Faculty of Medicine, Hacettepe University, Ankara, Turkey

T. Gurgan · A. Demirol
The Gurgan Clinic, 06540 Ankara, Turkey

D. Montjean
Service de médecine et biologie de la reproduction, 26 Boulevard de Louvain, 13285 Marseille, France

Y. J. R. Menezo (✉)
Laboratoire Clément, 17 Avenue d'Eylau, 75016 Paris, USA
e-mail: yves.menezo@gmail.com

Introduction

FSH is a key hormone in the regulation of follicular development. Together with the EGF network, these molecules mediate oocyte maturation and competence preceding the action of LH. Multidirectional communication between follicular cells is essential for oocyte maturation and competence [1]. This was clearly demonstrated by the work of Foote and Thibault [2] as early as 1969: premature disjunction between granulosa cells and the oocyte-cumulus complex prevents full oocyte competence; full competence requires a time-dependent sequence of events. However, the pathways through which hormonal and paracrine exchanges exert their effects on

oocyte competence are largely unknown. FSH isoforms, i.e. protein variants with a different glycosylation profile [3–5], regulate distinct biological pathways and have specific effects on granulosa cell function and ovarian follicular maturation [6–9]. Dynamic changes in FSH isoforms occur during the follicular cycle with short-living forms predominating in the pre-ovulatory phase, whereas long-acting molecules characterize the luteal-follicular transition [10, 11]. Such cyclical biological properties have been linked to the glycosylation status of the molecules, with half-life increasing with increase in sialic acid content [3, 4].

Several types of FSH isoforms with different glycosylation patterns [6] (recombinant and human-derived) are routinely used for ovarian stimulation [12–14], but very rarely with a dynamic modulation. A single product type with fixed isoform mix is used throughout the stimulation protocol. The benefit of these treatments is poorly understood in relation to effects on oocyte competence and clinical outcomes. Human derived FSH (hFSH) preparations are more highly sialylated (3 to 1) and more acidic, with a pH range from 3.0 to 5.2, [15] and they have a longer half life than recombinant FSH preparations. hFSH allows a longer pre-antral phase during stimulation, which appears to be more physiological: clinical and viable pregnancy rates for normo-ovulatory women are significantly higher than the rates for those who ovulate earlier in the follicular phase [16]. Acidic FSH induces E2 secretion more slowly, with onset of E2 rise only on day 4 and 5 [9]. Less glycosylated isoforms of FSH, which induce a higher level of estradiol secretion [17], may be required to sustain appropriate final maturation. In this study we investigated the effects of a more physiological stimulation protocol, starting with acidic hFSH (human-derived) followed by less acidic (recombinant) isoforms, and compared this to classical protocols that use a single type of FSH for stimulation. The impact on clinical data has been recorded. Cumulus cell transcript analyses were studied, as they are thought to represent a non-invasive approach for assessment of oocyte quality and molecular events triggered by FSH-based stimulation protocols [18–21]. Microarray technology offers a throughput transcript analysis tool to evaluate the effect of different FSH isoform mixes on biological and molecular pathways during ovarian stimulation. We investigated the cumulus cell transcriptome in response to ovarian stimulation protocols using recombinant FSH (rFSH), human-derived FSH (hFSH) or sequential hFSH + rFSH to map biological and molecular pathways activated by different FSH isoforms. The aim was to find proposed biomarkers that might be used to predict and screen for oocyte quality, with the implication that this could also predict embryo quality. These aspects will also be discussed with respect to sperm quality, especially its capacity to induce rapid oocyte activation [22–24]

Materials & methods

Patients

A total of 90 women undergoing treatment in an IVF program were randomised for three ovarian stimulation protocols, after pituitary down regulation with a GnRH agonist: (1) recombinant FSH (Puregon Pen, PP, rFSH), (2) human-derived FSH (hFSH, Fostimon) or (3) sequential combination of both (hFSH + PP). The latter consisted of 6 days administration of human-derived FSH followed by recombinant FSH for the remainder of the stimulation phase. The overall mean age of women included in this study was 30.2 years (±4.8), body mass index of 24.3 (±4.0), serum FSH level averaged 6.23UI/L (±1.67) and LH level 5.59UI/L (±4.87). No One of the patients had a PCOs profile according to the Rotterdam consensus. For all the patients, the fertilization of oocytes was performed using ICSI (intracytoplasmic sperm injection), in order to discard possible atretic and MI oocytes and to have a direct access to the cumulus cells, immediately post retrieval. The cells were processed on a percoll gradient for eliminating the blood cells, before freezing. Cumulus cells were collected for microarray analysis for the first 15 women, of each group, entering the protocol. The cumulus cells were pooled per each patient. The effect of the ovarian stimulation protocol on clinical outcome was evaluated using a bilateral *T*-Test.

Statistical analyses were performed using ANOVA and Chi square tests for comparison of percentages.

RNA purification

Total cumulus cell RNA was isolated using RNeasy Micro kit (Qiagen) following the manufacturer's instructions. Isolated RNA was eluted in 30 μl RNAse-free water. The purity and integrity of the total RNA preparation was verified using the Agilent 2100 Bioanalyser.

Microarray data analysis

Gene expression profile of cumulus cells was investigated by microarray analysis Affymetrix, HG-U133 Plus 2.0, see also [25]. All bioinformatic analyses of microarray data were performed as a service by GenoSplice technology (www.genosplice.com, Paris, France). For quality control, the expression distribution between chips was inspected at the probeset level by using the Relative Log Expression (RLE) and standard quality metrics (3′/5′ ratios, hybridization and labeling quality metrics). Microarray data from CEL files for all chips was normalized simultaneously and expression levels were generated using RMA from

Table 1 Patients biological values during treatment

Treatment	hFSH + PP	hFSH	PP	p
No of patients	30	30	30	
Mean age	28.9	27.1	30.1	0.03
BMI (kG/m2)	28.9	27.1	30.1	NS
basal FSH	6.8	6.7	6.1	NS (0.084)
basal LH	5.3	4.3	5	NS
basal E2	25.3	30.3	28.3	NS
Stimulation duration(d)	9	9.4	9.1	NS
Total FSH injected(IU)	2673	2850	2708	NS
E2 on hCG injection	1519	2294	2750	<0.001
(E2/MII oocyte retrieved)	**211**	**332**	**320**	
Endometrial thickness	11.1	10.8	10.9	NS

(ANOVA, F test)

PP Puregon Pen, *hFSH* Fostimon

Expression Console (Affymetrix, Santa Clara, CA). The normalized data was filtered for low-expressed probe sets. Only transcripts with a mean signal ≥5.5 in at least one treatment group were retained for further analysis. Differentially expressed transcripts were identified by one-way Anova (p-value≤0.05) and by unpaired T-Test (p-value≤0.05 and fold-change≥1.5). Mev4.0 software from TIGR (The Institute of Genome Research) was used to perform hierarchical clustering in order to cluster amongst the gene signal intensities and among the samples.

Results

Clinical outcome

The sample size was not calculated for power in advance, consequently we found no statistical difference in clinical outcome between the three protocols ($p>0.05$). hFSH, PP and hFSH + PP revealed no detectable difference with respect to impact on follicular growth, oocyte maturation, fertilization rate, endometrial thickness, implantation rate and pregnancy rate. No cycles were cancelled due to overstimulation and resulting OHSS. The results are summarized in Tables 1 and 2 a significant difference was observed vs. age between the three groups (0.03). The most striking difference being in the level of estrogens at the time of hCG injection to trigger ovulation. The sequential treatment showed the lowest level of E2 per MII oocyte retrieved (211) much lower than that observed in the two other treatments (hFSH: 332, PP: 320). In terms of parameters related to the in vitro procedures, the only marginal significance seen was for fertilization rates (better for hFSH alone $p=0.047$)

Table 2 ART parameters of the patients. ANOVA F test, for the comparison of means, Chi2 for the comparison of percentages

Treatment	HFSH + PP	hFSH	PP	p values
No of patients	30	30	30	
No MII	7.2	6.9	8.6	NS,
Mean No of 2 PNs	6.4	6.5	7.6	NS,
Fertilization rate(%)	88.9	94.7	88.4	0.047
Cleavage on day2(%)	100	97.9	100	NS
Gade1 embryos(%)	62	53.9	60.7	NS
Grade2 embryos	38	45.6	39.3	NS
Grade3 embryos	X	0.5	X	
Mean No of embryos tfrd	2.4	2.7	3.1	0.004
Gestational sacs	17	16	14	NS
Sacs with cardiac activity	16	16	11	NS
Pregnant patients	13	12	11	NS
Pregnancy rates (%)	43.3	40	36.6	NS
Implantation rates (%)	22.2	20	11.8	NS (0.17)
Ongoing pregnancies (rates %)	12 (40)	11 (36.6)	8(26.6)	NS (0.14)
Take home baby (rates %)	11 (36.6)	11(36.6)	7(23.3)	NS
Patients with embryos frozen(%)	12(40)	12(40)	11(36.6)	NS
Total no of embryos frozen	56	74	60	
Cryo cycles	8	7	6	
Ongoing pregnancies	4 (50 %)	2 (28.6 %)	2 (33 %)	NS

Table 3 Expression variations hFSH vs PP, level of significance $p<0.01$

hFSH vs. PP				
Gene symbol	*T*-test *P*-value	Fold-change	Regulation	Gene title
HTRA1	1,72E-03	1.89	Up	HtrA serine peptidase 1
SERPINE2	6,42E-03	1.88	Up	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
EIF4E2	3,47E-03	1.71	Up	Eukaryotic translation initiation factor 4E family member 2
FOSB	6,97E-03	1.66	Up	FBJ murine osteosarcoma viral oncogene homolog B
CRTAP	7,77E-03	1.63	Up	Cartilage associated protein
FKBP11	4,12E-04	1.62	Up	FK506 binding protein 11, 19 kDa
ALDH2	7,19E-03	1.56	Up	Aldehyde dehydrogenase 2 family (mitochondrial)
ANP32A	2,44E-03	1.55	Up	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
ARRDC4	6,64E-03	1.65	Down	Arrestin domain containing 4
RPS26	9,85E-04	1.62	Down	Ribosomal protein S26
MLLT3	2,53E-03	1.54	Down	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3

The following genes:SF3B1,LAMP1,SFRS6,ATP1B3,LAPTM4B,SMARCA1, TMEM97,RCHY1,CD24,RAC1,HMBG2,USP1 are up-regulated at a level $0.05<p<0.01$

The chi square test reveals no significant differences between treatments: implantation rate (total fetal cardiac activity/ total transferred embryos), *p*-value (general)=0.140 (no statistically significant difference). A significantly higher number of embryos was transferred in the PP group. The pregnancy rates, obtained with transfers of fresh embryos were not different between the groups hFSH + PP vs. hFSH alone, $p=0.230$; hFSH + PP vs. PP alone, $p=0.052$; hFSH vs. PP $p=0.474$. The cumulated (Fresh + frozen) embryo transfers did not show any significance. hFSH + P vs. hFSH alone, $p=0.605$; hFSH + PP vs. PP alone, p-=0.302; hFSH vs. PP, $p=0.605$

Microarray analysis

Microarray analysis revealed 579 regulated genes when comparing the ovarian stimulation regimens. Unpaired *T*-Test and one-way Anova analysis of microarray data (*p*-value≤0.05, fold-change ≥1.5 and *p*-value≤0.05) highlighted differential expression of genes. Gene symbols, fold changes and p-values are summarized in Tables 3, 4 and 5. In term of Clusters (Table 6), the ones involved in specific biological i.e. processes, molecular function and pathways show also important modifications. The sequential regimen (hFSH + PP) significantly influenced nucleoside, nucleotide and nucleic acid metabolism and mRNA transcription to a greater extent than PP ($p=7.53e^{-5}$ and $1.76e^{-4}$). Furthermore, hFSH + PP increases cholesterol metabolism significantly when compared with hFSH only ($p=4.01e^{-3}$, Table 3, 4 and 5). Biological processes, molecular function and regulated pathways, in relation to ovarian stimulation protocol and p-values are described in Table 6. hFSH activates the following pathways: cell morphology ($p=8.6e^{-4}$), cell death ($p=1.84e^{-3}$), cell growth and proliferation ($p=2.6e^{-3}$) and embryonic development ($p=1.3e^{-3}$). hFSH enhances the expression of genes involved in regulation of cell adhesion (CD24), proliferation, differentiation, and transformation: *FOSB*, *SRSF6* and *USP7*, ($p=2.5e^{-3}$). PP preferentially activates cell growth and proliferation ($p=2.7e-3$), cell death ($p=2.28e-2$) and protein synthesis (1.45e-2).

Transcripts and pregnancy

Although we found no correlation between ovarian stimulation protocol and clinical outcome, we were able to identify transcripts that were regulated in cumulus cells surrounding oocytes leading to a successful pregnancy (Table 7). 17 transcripts proved to be regulated in competent oocytes, 14 with a down-regulated expression and three with an increased expression. Gene symbol, fold changes and *p*-values are summarized in Table 7.

IPA analysis revealed that four major biological functions are down-regulated in cumulus cells surrounding competent oocytes, namely: cell death ($p=2.14e^{-2}$), cholesterol metabolism ($p=7.7e^{-5}$), cell cycle progression ($7.4e^{-3}$) and cellular morphology ($1.05e^{-2}$); only one, a vitamin metabolism pathway, was up-regulated ($1.2e^{-4}$)

Table 4 Expression variations hFSH + PP vs hFSH alone. Limit of significance $p<0.01$

hFSH + PP vs. hFSH				
RPS26	6,33E-08	2.10	Up	Ribosomal protein S26
ARRDC4	6,62E-04	1.96	Up	Arrestin domain containing 4
AK3L1	9,39E-03	1.70	Up	Adenylate kinase 3-like 1
IL8	1,88E-06	3.16	Down	Interleukin 8
FAM110C	9,17E-03	2.14	Down	Family with sequence similarity 110, member C
CYP51A1	1,36E-03	1.95	Down	Cytochrome P450, family 51, subfamily A, polypeptide 1
NAP1L5	1,49E-03	1.90	Down	Nucleosome assembly protein 1-like 5
C19orf12	3,82E-04	1.86	Down	Chromosome 19 open reading frame 12
TANC1	3,70E-03	1.85	Down	Tetratricopeptide repeat, ankyrin repeat
HMGCR	1,39E-04	1.76	Down	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
KCMF1	1,11E-03	1.71	Down	Potassium channel modulatory factor 1
RBM25	9,56E-04	1.70	Down	RNA binding motif protein 25
RCHY1	2,63E-03	1.60	Down	Ring finger and CHY zinc finger domain
ILF3	2,41E-03	1.60	Down	Interleukin enhancer binding factor 3, 90 kDa
ADIPOR2	9,05E-03	1.59	Down	Adiponectin receptor 2
BAT2D1	1,87E-03	1.57	Down	BAT2 domain containing 1
WBP11	6,27E-04	1.57	Down	WW domain binding protein 11
SLC30A7	2,19E-05	1.56	Down	Solute carrier family ZN transporter
HIVEP3	2,44E-03	1.55	Down	Human immunodeficiency virus type I enhancer binding protein 3
CUL4B	6,22E-03	1.54	Down	Cullin 4B
EIF5	3,64E-03	1.54	Down	Eukaryotic translation initiation factor 5
USP12	4,51E-05	1.54	Down	Ubiquitin specific peptidase 12
KIAA1377	1,83E-04	1.54	Down	KIAA1377
ZNF275	1,61E-03	1.53	Down	Zinc finger protein 275
GPR56	5,46E-04	1.53	Down	G protein-coupled receptor 56

The following genesAKAP2, Amigo2, HSPA1, TFRC, RSG2, WTAP, DR1, IGF1R, HTRA1 are down regulated at a significance level $0.05<p<0.01$
Note the strong down regulation of the Zinc transporter SLC30A7 (see [38])

Discussion

A sequential system for controlled ovarian stimulation (COH) is closer to natural physiology, in that there is a higher number of isoforms in the mix. Our results showed that this strategy led to a trend towards a higher, even if not significant pregnancy rate, as mentioned earlier [26–28]. The use of hFSH yields the same results as the use of recombinant FSH, in agreement with several other authors [26, 27]. Cost effectiveness is an interesting comparison. The sequential administration of hFSH followed by rFSH is slightly more efficient and a more physiological treatment In term of metabolic and hormonal modifications, hFSH seems to be exactly in the middle compared to sequential and recombinant alone. In our hands sequential stimulation leads to lower estradiol levels, as would be expected in consideration of differences in glycosylation, and in contrast to what has been reported elsewhere [26, 27] The E2 level per MII oocyte appears to be far less important in the hFSH + PP regimen than in the other treatments. A level of circulating E2 that is more physiological may also have a beneficial effect on implantation, as often mentioned [29].

The factors that contribute to making a good embryo is a recurrent question; as advocated earlier [30], the oocyte/embryo in not necessarily the cause of failure; poor embryo transfer quality and an hostile uterine environment are also causes of recurrent failing. In IVF the yield per oocyte collected is well below 10 % [31], and it is hard to believe that the oocyte is the primary reason for this. Transcriptome analysis of cumulus cells has been proposed as a non-invasive tool for determining the quality of the corresponding oocyte, but this raises a few questions [18, 19, 32].

First, misinterpretations and pitfalls [30] must be avoided: rapid cleavage cannot be a suitable parameter for assessment, as it depends mainly the paternal

Table 5 Expression variations (FSH + PP) vs PP Level of significance $p<0.01$

hFSH + PP vs. PP				
ALDH2	1,74E-03	1.69	Up	Aldehyde dehydrogenase 2 family (mitochondrial)
RAC1	6,58E-03	1.68	Up	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
PRKAR2B	1,06E-03	1.67	Up	Protein kinase, cAMP-dependent. Regulation of cAMP signalling
ADD3	9,51E-03	1.65	Up	Adducin 3 (gamma)
SSBP2	8,98E-03	1.62	Up	Single-stranded DNA binding protein 2
SMARCA1	8,78E-03	1.58	Up	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1
SUZ12	8,90E-03	1.55	Up	Suppressor of zeste 12 homolog (Drosophila)
LOC572558	7,37E-04	1.51	Up	Hypothetical locus LOC572558
RBP1	5,21E-03	1.51	Up	retinol binding protein 1, cellular
ANP32A	4,59E-03	1.50	Up	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
ATP5A1	1,89E-03	1.50	Up	ATP synthase, H + transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
AKAP2	9,89E-05	2.43	Down	A kinase (PRKA) anchor protein 2
CYP51A1	1,25E-03	2.02	Down	Cytochrome P450, family 51, subfamily A, polypeptide 1
GRK5	3,15E-03	1.85	Down	G protein-coupled receptor kinase 5
TET3	4,51E-03	1.80	Down	Tet oncogene family member 3
MLLT3	5,38E-03	1.76	Down	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3
WSB2	8,36E-04	1.67	Down	WD repeat and SOCS box-containing 2
TM4SF1	3,12E-03	1.65	Down	Transmembrane 4 L six family member 1

The following genes AK3L1,FOSB, NAP1L1, EGR3,ESD,ZnF664,TMEM97,CRTAP,BNIP3,VSP41,FKBP11,NCOA4, TPI1 and EIF4E2 are up regulated at $0.05<p<0.01$

IL8 and TM4SF1 genes are down regulated at $0.05<p<0.01$

contribution [23, 24]: the paternal genome affects embryonic development between pronuclear formation and 2-cell stage before embryonic genome activation. Early cleavage or "early embryo morphology aspect" as a test for oocyte competence is highly unreliable and is therefore questionable. This strongly limits the significance of studies that associate CC mRNA levels and early embryo cleavage [33]. The exact opposite concerning rapid cleavage and embryo quality has been demonstrated in mouse: Faster development rates in vitro can be correlated with loss of genomic imprinting [34]. So far, the only accurate approaches for analysis are implantation and delivery rates. Apart from the impact of the uterine environment, the paternal effect can have an impact on the capacity for DNA repair in the young embryo [35] and its translational capacity through mRNAS [36]. This limits the significance of some observations.

It is generally assumed (guessed?) that there is a positive correlation between CC gene expression and oocyte quality. Moreover, there are large variations in the number of "sensible" specific gene markers for ongoing pregnancy, from four [21] to 630, and then 45 for the same group, the majority being up-regulated [18, 19]. It is not clear that a high level of expression in CC at this stage is a sign of quality: the cumulus cells have reached the end of their life's journey, especially after cumulus expansion. Their trophic role towards the oocyte is ending, if not completed: this fits with the expression of AREG, one of the LH mediators, which is fully down regulated in CC cells at the time of oocyte recovery [37]. This is also especially true if we consider the Metallothioneins (MT) and metal responsive transcription factors (MTFs) [38]. The importance of MTs in supporting early embryo development in human embryos is also emphasized by the transcriptome profile of sperm in cycles that lead to pregnancy after ICSI [39]. MTFs, especially MTF2, are highly expressed in oocytes and are completely absent in cumulus cells collected at the time of oocyte retrieval, when the transfer

Table 6 Biological process, molecular function and pathway activated regarding ovarian stimulation protocol

Biological process	*P*-value	Molecular function	*P*-value	Pathway	*P*-value
hFSH vs. PP					
Proteolysis	1,78E-03	mRNA splicing factor	4,35E-03	p38 MAPK pathway	1,39E-03
Protein metabolism and modification	5,20E-03	mRNA processing factor	8,47E-03		
mRNA splicing	1,73E-02	Nucleic acid binding	4,51E-02		
Pre-mRNA processing	3,06E-02				
hFSH + PP vs h FSH					
Cholesterol metabolism	4,01E-03	Hsp 70 family chaperone	2,04E-04	Apoptosis signaling pathway	1,41E-02
Protein complex assembly	4,01E-03	Chaperone	2,45E-02		
Protein metabolism and modification	6,43E-03	Other RNA-binding protein	2,88E-02		
Proteolysis	9,67E-03	Other G-protein modulator	4,28E-02		
Cell surface receptor mediated signal transduction	2,30E-02				
Steroid metabolism	2,63E-02				
Protein folding	2,71E-02				
Stress response	3,10E-02				
Apoptosis	3,63E-02				
Signal transduction	3,73E-02				
hFSH + PP vs. PP					
Nucleoside, nucleotide and nucleic acid metabolism	7,53E-05	Phosphatase inhibitor	2,47E-05	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	4,76E-03
mRNA transcription	1,76E-04	Phosphatase modulator	1,30E-04	p38 MAPK pathway	5,47E-03
mRNA transcription regulation	1,46E-03	Select regulatory molecule	1,62E-03	Inflammation mediated by chemokine and cytokine signaling pathway	2,17E-02
DNA replication	3,19E-03	Nucleic acid binding	1,90E-03		
DNA metabolism	4,76E-03	Translation initiation factor	6,03E-03		
Developmental processes	6,16E-03	Other miscellaneous function protein	8,59E-03		
Cholesterol metabolism	7,43E-03	Other G-protein modulator	1,05E-02		
Chromatin packaging and remodeling	1,02E-02	Translation factor	1,79E-02		
Cell structure and motility	2,04E-02	Other membrane traffic protein	2,08E-02		
Signal transduction	2,20E-02	Transcription factor	3,70E-02		
Protein phosphorylation	3,57E-02	Transcription cofactor	4,03E-02		
Cell cycle	4,10E-02	G-protein modulator	4,11E-02		
Cell proliferation and differentiation	4,38E-02	Isomerase	4,47E-02		
Ligand-mediated signaling	4,52E-02				
Steroid metabolism	4,70E-02				

of material between the CC cells and the oocyte has been completed: "the job has been done". The sequential protocol decreases also SLC30A7, a Zn transporter (Table 4). It is interesting to note that hFSH treatments appears to be rather associated with depletion, rather than over-expression of Poly(A) mRNAs. hFSH + rFSH strongly down-regulates IL8 (Table 4). Indeed, when we compared RNA expression in CC from cumuli leading to pregnancy (20 pregnant patients vs. 25 not pregnant) we found 13 genes that were depleted: *FOLR1*, *SRP72*, *SYNCRIP*, *OVOS2*, *ITGB3BP*, *APOC1*, *VPS28*, *GM2A*, *CYP1B1 (*metabolism of lipid and steroids*)*, *TAF10*, *DEPDC6*, *PGLS*, *HSD17B1 (*a cluster in the synthesis of cholesterol and further on, of steroids*)* and *KCTD21.* Three genes were more highly expressed in the pregnant than the non-pregnant group*: TMEM39A*, *LOC92497* and *MOCOS* (Table 7). One striking gene of note is FOLR1: It binds folic acid and its reduced derivatives, and transports 5-methyltetrahydrofolate into cells. The human oocyte has a very high expression of FolR1, as well as SLC19A1 [40], Solute carrier family 19 (folate transporter), member 1, indicating a huge level of trafficking around this

Table 7 Significant variations in mRNA expression between pregnant vs. non pregnant groups

Gene symbole	*P*-value (pregnant vs. not)	Fold (pregnant vs. not)	Regulation	Gene title	Gene ID
TMEM39A	5,34E-03	1.98	Up	Transmembrane protein 39A	55254
LOC92497	2,91E-03	1.65	Up	Hypothetical LOC92497	92497
MOCOS	4,33E-02	1.52	Up	Molybdenum cofactor sulfurase	55034
FOLR1	1,88E-02	2.04	Down	Folate receptor 1 (adult)	2348
SRP72	1,82E-02	1.83	Down	Signal recognition particle 72 kDa	6731
SYNCRIP	1,72E-02	1.74	Down	Synaptotagmin binding, cytoplasmic RNA interacting protein	10492
OVOS2	3,40E-02	1.68	Down	Ovostatin 2	100132881
ITGB3BP	4,08E-02	1.68	Down	Integrin beta 3 binding protein (beta3-endonexin)	23421
APOC1	1,50E-02	1.60	Down	Apolipoprotein C-I	341
VPS28	1,56E-02	1.59	Down	Vacuolar protein sorting 28 homolog (S. cerevisiae)	51160
GM2A	8,85E-03	1.56	Down	GM2 ganglioside activator	2760
CYP1B1	1,12E-02	1.54	Down	Cytochrome P450, family 1, Lipid metabolism	1545
TAF10	1,25E-02	1.54	Down	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30 kDa	6881
DEPDC6	3,43E-02	1.53	Down	DEP domain containing 6	64798
PGLS	1,66E-02	1.51	Down	6-phosphogluconolactonase	25796
HSD17B1	4,54E-02	1.51	Down	Hydroxysteroid (17-beta) dehydrogenase 1	3292
KCTD21	1,37E-02	1.51	Down	Potassium channel tetramerisation domain containing 21	283219

molecule. Folic acid is involved in methylation and therefore imprinting through homocysteine recycling, one of the cell's universal poisons. Folic acid also helps in protecting cells from oxidative stress and free radicals in general. One must not forget its major role in methylation of Uracil into Thymine at the time when DNA synthesis is of major importance. The timing of this down-regulation is probably an important paradigm. An abnormally low endogenous folate pool will lead to abnormal imprinting and later may play a role in the development of autism [40–42], especially as the early embryo is poorly able to recycle homocysteine through the CBS pathway [40]. Down-regulation of OVOS 2, ovostatin having Serine-type endopeptidase (chymotrypsin-like) inhibitor activity is also interesting, with respect to sperm swelling immediately post fertilization.

The relationship between mRNA expression in the CC and oocyte quality is in conclusion rather disappointing. Available results are difficult to handle, and no clear message can be extracted. The feasibility of such a technique as a tool to select the best oocytes remains to be established on a large scale. It seems obvious that variations in the transcriptome of the CC cells are highly dependent upon the patients' genotype, and its usefulness for selecting the best embryos is, at the very least, questionable.

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