

Identification of follicular marker genes as pregnancy predictors for human IVF: new evidence for the involvement of luteinization process

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ABSTRACT: Multiple pregnancy represents an important health risk to both mother and child in fertility treatment. To reduce a high twin rate, restriction to one embryo transfer is needed. Morphological evaluation methods for predicting embryo viability has significant limitations. Tight communication exists between the follicular cells (FCs) and the oocyte; therefore, developmental competence may be determined by markers expressed in the surrounding FCs. In this study, cells were recovered on a per-follicle basis by individual follicle puncture. Hybridization analysis using a custom-made complementary DNA microarray containing FC transcripts was performed. Genes expressed in FCs associated with good morphological transferred embryos were identified from follicles associated with a pregnancy outcome (pregnancy group) or no pregnancy (non-pregnancy group). Ten candidates from the Pregnancy group and three from the Non-pregnancy group were validated by quantitative RT–PCR. The best predictors associated with pregnancy were UDP-glucose pyrophosphorylase-2 and pleckstrin homology-like domain, family A, member 1. Genes assessment showed no significant candidate genes associated with non-pregnancy outcome, but GA-binding protein transcription factor $\beta 1$ showed a tendency to be potentially more expressed in the non-pregnancy group. These markers could be related to granulosa luteinization process and could be used to improve embryo selection for successful single embryo transfer.

Key words: embryo quality / follicular cells / granulosa / IVF / gene expression

Introduction

In vitro fertilization is one of the most common treatments of infertility. This technique usually involves the implantation of more than one embryo to maximize the possibilities of a viable pregnancy. Unfortunately, the transfer of multiple embryos increases the risk of multiple pregnancy associated with potential harmful consequences to the mother and the babies (Westergaard *et al.*, 1999; Schieve *et al.*, 2002; Pinborg, 2005) including high risks of prematurity, low or very low birthweight and perinatal death. To alleviate this problem, there is growing evidence for the need to prevent multiple pregnancies by the selection of a high-quality embryo to perform single embryo transfer.

Until now, non-invasive embryo selection has been based mainly on morphological and developmental embryo scoring criteria such as early cleavage rate, appearance of pronuclei, fragmentation rate, number of blastomeres and embryo symmetry (Van Royen *et al.*,

2001; De Neubourg *et al.*, 2004; Weghofer *et al.*, 2008). However, the efficiency of current methods for predicting embryo viability has significant limitations (Guerif *et al.*, 2004) and needs further improvement. Many studies have demonstrated that embryos with satisfactory morphological appearance can show chromosomal abnormalities (Munne *et al.*, 1995; Gianaroli *et al.*, 1999; Munne, 2006) and consequently do not lead to pregnancy. Furthermore, it has been reported that some ovarian stimulation protocols affect ploidy in comparison to other protocols (Weghofer *et al.*, 2008). Therefore, additional non-invasive strategies for identifying the ideal embryo for transfer are needed to increase the sensitivity and objectivity of embryo selection.

Recently, several studies in human were undertaken to identify the gene profile of granulosa and cumulus cells to evaluate their predictive potential regarding embryo quality (McKenzie *et al.*, 2004; Zhang *et al.*, 2005; Assou *et al.*, 2006; Cillo *et al.*, 2007; Hamel *et al.*, 2008, 2010; van Montfoort *et al.*, 2008; Adrianenssens *et al.*, 2010). Other technologies, like proteomics, respirometry and others are also

investigated (for review: Lopes *et al.*, 2007; Singh and Sinclair, 2007). Morphological embryo evaluation, and these potential alternatives could be complementary to genomics to assess embryo quality.

It has been well established that an intricate system of bi-directional communication between oocyte and follicular cells (FCs) occurs throughout follicular development (Buccione *et al.*, 1990; Eppig *et al.*, 2002; Gilchrist *et al.*, 2004) and is crucial for the acquisition of developmental competence by the oocyte (de Loos *et al.*, 1991; Webb *et al.*, 2002). The oocyte plays a dynamic role in controlling important metabolic processes (Sugiura *et al.*, 2005) as well as granulosa cell proliferation and differentiation (Eppig, 2001), and depends on FCs for its growth, development, meiosis and global transcriptional activity (De La Fuente and Eppig, 2001; Eppig, 2001; Sugiura *et al.*, 2005) to acquire full developmental potential. Also, as evidenced by ovarian stimulation protocols used in IVF procedures, FSH and LH surges promote important changes in gene expression of FCs (Friedmann *et al.*, 2005; Grondahl *et al.*, 2009; Weghofer *et al.*, 2008) and can in turn indirectly influence oocyte maturation, ovulation (Tounoun *et al.*, 2001) and luteal development (Devoto *et al.*, 2002). This clearly indicates that FCs surrounding the oocyte can profoundly influence the quality of the oocytes recovered in IVF procedure and, in consequence, the quality of embryos obtained.

Recently in our lab, the identification of potential markers associated with oocyte competence in late folliculogenesis after the pre-ovulatory LH surge has been accomplished in groups of patients who had a successful pregnancy (Hamel *et al.*, 2008). These candidate markers were thereafter evaluated in FCs from two embryos of individual patients with successful pregnancy (Hamel *et al.*, 2010). In these previous papers, the comparison was not done among good morphology embryos but between good and rejected embryos. In this paper, the investigation was pushed further and we have recovered new samples and made a new hybridization to compare embryos that fulfilled all the morphological characteristics to achieve embryo transfer, but led to a successful or unsuccessful pregnancy outcome. For clinical application relevance, these potential markers could be used as indicators to successfully discriminate embryos presenting similar good morphological evaluation with the objective of successful single embryo transfer.

Methodology

FCs were obtained from women undergoing IVF treatments at the Fertility Center at the Ottawa Hospital, Ontario, Canada. Women ($n = 18$) (average age of 36 years old) with major indications for IVF, such as tubal infertility, unexplained infertility including endometriosis Stage I/II/III were recruited for the study. Patients with polycystic ovary syndrome, or partners with severe male factors requiring ICSI were not included in the study. It was the first IVF procedure for 17 patients and the second IVF procedure for one patient. The procedure was performed with the approval of the Ottawa Hospital Research Ethics Board.

Following ovarian stimulation, follicular fluid, FCs and oocytes from individual follicles were collected 36 h after hCG administration by ultrasound-guided follicular aspiration using a double lumen needle. Average patient's level of estradiol at the time of hCG injection was 5 873.43 IU (SD = 2641.03). The oocytes and surrounding cumulus cells were removed for IVF procedure. FCs recovery was performed as described previously (Hamel *et al.*, 2008). Cells present in the follicular fluid recovered are mainly granulosa cells (Hamel *et al.*, 2008). After the recovery procedure,

cells were rapidly frozen and stored in liquid nitrogen until RNA extraction.

Treatment assignment

Data (fertilization, embryo development, embryo morphology, transfer and pregnancy) generated from each follicle was recorded by an embryologist. Each embryo was scored according to the clinic's embryo selection protocol and based on main criteria, the cleavage stage and morphological characteristics (shape, size, granularity and 3D orientation of the blastomeres and space inside the zona pellucida occupied by enucleated fragments). Fresh transferred embryos were at least 6–7 cells with high scores in morphology grade. Depending on the IVF protocol used, one or two embryos were transferred on either Day 3 (16 patients) or Day 5 (2 patients). Pregnancy was confirmed by the presence of a fetal heart-beat by ultrasound at 6–8 weeks.

For the hybridization experiments, we selected seven patients who produced FCs from oocytes that resulted in a 100% pregnancy success rate (positive samples; $n = 9$ follicles) and seven patients who produced FCs from oocytes that resulted in a transferred embryo with unsuccessful pregnancy (negative samples; $n = 9$ follicles). For qRT-PCR, three additional pools of follicles [Pool 1 (3 patients; $n = 3$ follicles), Pool 2 (4 patients; $n = 4$ follicles) and Pool 3 (4 patients, $n = 4$ follicles)] were created from FCs associated with 100% pregnancy success rate, and were called pregnancy Groups 1, 2 and 3, respectively. In pregnancy groups, all double embryo transfers resulted in a double pregnancy (twin) and a single embryo transfer resulted in a twin pregnancy for one patient in Pool 1. Three other pools [Pool 1 (3 patients; $n = 3$ follicles), Pool 2 (4 patients; $n = 4$ follicles) and Pool 3 (4 patients, $n = 4$ follicles)] were assigned to the non-pregnancy Groups 1, 2 and 3, respectively, containing FCs resulting in transferred embryos with unsuccessful pregnancy (Table I).

RNA isolation

Total RNA from FCs was extracted with 1 ml of Trizol reagent (Invitrogen, Burlington, Canada) following the manufacturer's protocol. RNA was then further purified using the RNeasy total RNA clean-up protocol with DNase treatment (Qiagen, Mississauga, ON, Canada). The concentration and integrity of the RNA samples were assessed spectrophotometrically at 260 nm and on an Agilent 2100 Bioanalyzer (Agilent Technology Inc., Santa Clara, CA, USA) running an aliquot for the RNA samples on the RNA 6000 Nano LabChip. RNA was reverse transcribed to cDNA for hybridizations and qRT-PCR experiments.

Microarray hybridizations

Microarray hybridizations were performed with our custom-made cDNA array slides previously elaborated (Hamel *et al.*, 2008). Total RNA of FCs was amplified using the RiboAmpT7 RNA Amplification kit (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions. The RNA was submitted to one round of amplification and the quantity of aRNA was estimated by spectrophotometry at 260 nm. Probes were labelled with the ULSTTM aRNA Fluorescent Labelling Kit (Kreatech Biotechnology, Salt Lake City, UT, USA) according to the manufacturer's protocol, but without the aRNA fragmentation step. Slides were hybridized overnight at 50°C with labelled purified probes using the SlideHyb #1 buffer (Ambion, Austin, TX, USA). Hybridizations were performed in a SlideBooster using the Advacard AC3C (The Gel Company, San Francisco, CA, USA). Slides were then washed twice with $2 \times$ SSC/0.5% SDS for 15 min at 50°C and twice with $0.5 \times$ SSC/0.5% SDS for 15 min at 50°C.

The experimental design included dye-swap replication to account for dye incorporation bias. Hybridization was performed using a positive group and a negative group. The RNA from both positive and negative

Table I Treatment assignment with follicular cell tissues and patient characteristics for qRT–PCR validation.

	Average number of oocytes recovered	Average number of oocytes fertilized	Average number of embryos transferred	Pools	Patients	Embryo transfer	Pregnancy outcome
Pregnancy groups	6.00 ± 1.344	3.33 ± 0.9428	1.22 ± 0.1470*	Pool 1	1	1	Single
					2	1	Single
					3	1	Twins
				Pool 2	1	1	Single
					2	1	Single
					3	2	Twins
				Pool 3	1	1	Single
					2	1	Single
					3	2	Twins
Non-pregnancy groups	6.89 ± 0.9782	4.00 ± 0.8498	1.89 ± 0.1111*	Pool 1	1	1	No Pregnancy
					2	2	No Pregnancy
					3	2	No Pregnancy
				Pool 2	1	2	No Pregnancy
					2	2	No Pregnancy
					3	2	No Pregnancy
				Pool 3	1	2	No Pregnancy
					2	2	No Pregnancy
					3	2	No Pregnancy

*Statistically different ($P < 0.01$).

groups was used as probes in a dye-swap manner. Slides were scanned using the VersArray ChipReader System (Bio-Rad, Mississauga, ON, Canada) and analysed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, Bethesda, MA, USA). Determination of the background signal threshold was performed with the SpotReport cDNA controls (Stratagene, La Jolla, CA, USA), which determine the background ($t = m + 2 \times SD$, where ‘ t ’ is the calculated threshold, ‘ m ’ the mean and ‘ SD ’ the standard deviation of the negative control data, $n = 58$). Transcripts above the threshold were considered as present in granulosa cells, whereas the other transcripts were eliminated from the analysis. Fluorescence signal intensities for each replicate were \log_2 -transformed, normalized by the Loess method, and corrected for background. Microarray data is publically available at <http://www.ncbi.nlm.nih.gov/geo/> Accession number GSE22869.

Candidate gene selection

Selection of clones for further analysis was based on the microarray results obtained in this experimentation and from the custom-made cDNA array slides and analysis performed with other hybridizations described previously (Hamel et al., 2008). Markers were selected and graded according to their number of occurrences in different libraries, their repetition in the same library and signal intensity.

Quantitative PCR

Primers for each candidate gene were designed with the Primer3 web interface using sequences derived from the National Center for Biotechnology Information (NCBI) corresponding to our library sequences (Table II). Real-time analysis measured and compared the three different groups of FCs for the pregnancy and non-pregnancy groups with the same procedure already published (Vigneault et al., 2004; Hamel et al., 2008). Briefly, for each sample, reverse transcription was performed using 50 ng of granulosa cell RNA using the Sensiscript kit (Qiagen) according to the manufacturer’s directions. To confirm that the right product was amplified, all amplifications were visualized on an agarose gel (2%) and then sequenced. For each marker, a standard curve, consisting of purified PCR products and quantified with a spectrophotometer

(NanoDrop ND-1000, NanoDrop Technologies), was included in the run. Four standards of the purified PCR products were diluted to perform a standard curve. qRT–PCR was performed on a LightCycler device (Roche Diagnostics) using SYBR green incorporation for real-time monitoring of amplicon production. Only one peak could be seen in melting curve analysis, demonstrating specific amplification of the PCR product. Three housekeeping genes [β -actin (ACTB), cyclophilin A (PPIA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were used as internal controls (previously described in Hamel et al., 2010).

Statistical analysis

Analysis of gene expression stability over the different positive and negative samples was performed using the geNorm VBA applet software (Vandesompele et al., 2002). This analysis relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples, regardless of the experimental condition or cell type, and determined as the standard deviation of the logarithmically transformed expression ratios. Using the software, the internal control gene stability (the M value) was calculated as the average pair wise variation of a particular gene (ACTB, PPIA and GAPDH in this study) with respect to the rest of the genes, and ranking was made based on these values. The most stable reference genes were identified by stepwise exclusions of the least stable gene and recalculating the M values. Following GeNorm analysis, actin and GAPDH were the most stable genes, with M values of < 1.5 as per software recommendation (M values = 0.634). Normalization of genes was calculated according the normalization factors for each sample. Data are presented as mean \pm SEM. Evaluation of mRNA differences between the positive and negative groups was performed by a non-parametric two-tailed unpaired t -test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$) and a tendency at the 90% level ($P < 0.1$).

Results

Data generated from each follicle (fertilization, embryo development, embryo morphology, transfer and pregnancy) were recorded by an

Table II Sequences of specific primers used for amplification in qRT-PCR.

Genes	Average fold change HYB A ^a	Average fold change HYB B ^b	Primer sequences	GenBank accession number	UniGene accession number	Product size (bp)	Annealing temperature (°C)	Fluorescence acquisition temperature (°C)
Genes associated with competent follicle								
<i>EREG</i>	2.55	1.81	Up 5'-CTGGGTTTCCATCTTCTACAGG Low 5'-GCCATTTCATGTCAGAGCTACAC	NM_001432	Hs.115263	159	56	82
<i>DPYSL3</i>	4.43	1.59	Up 5'-CAAAGTCAGTCTTGAGCAGAGG Low 5'-AGAAGCAGCATGATAGGGAAAG	NM_001387	Hs.519659	253	55	84
<i>PGR</i>	2.79	1.69	Up 5'-GATTGAGAAGCCAGCCAGAG Low 5'-TACCTTCCATTGCCCTCTTAAA	NM_000926	Hs.32405	149	54	82
<i>YWHAZ</i>	3.02	1.61	Up 5'-GGTGATGACAAGAAAGGGATTG Low 5'-GCGTGCTGTCTTTGTATGACTC	NM_145690	Hs.492407	244	54	82
<i>MARCKS</i>	2.99	1.62	Up 5'-TTCTTCCTCTGCCTTGTTCCTC Low 5'-CAGCCTTTACACCATTTCTAGTG	NM_002356	Hs.519909	247	56	80
<i>UGP2</i>	2.98	1.61	Up 5'-GACTCAGTCGCACCAAGTTTC Low 5'-TTCCTCTCCTGCTTAAACAACC	NM_006759	Hs.516217	163	55	77
<i>SEMA3A</i>	2.22	2.01	Up 5'-CAGCCCTGAAGAGAGAATCATC Low 5'-GCTCTGTGTCAATGACTTCCAG	NM_006080	Hs.252451	281	55	83
<i>LRP8</i>	2.24	2.60	Up 5'-CCAGAGTGCTCAGAACTCAAG Low 5'-CCATCTTTATCTTCATCCACAGC	NM_004631	Hs.576154	258	54	82
<i>PIR</i>	4.33	1.54	Up 5'-AAGCCAGTAAGGATGGTGTGAC Low 5'-AAGACAAAGTGGCTTCTCTTGG	NM_003662	Hs.495728	298	55	83
<i>PHLDA1</i>	2.12	1.56	Up 5'-AGCTCAATAACTGTTGGGACAAAG Low 5'-CATGTGAGGGAAACTCCTTTAAGT	NM_007350	Hs.602085	421	55	81
Genes associated with non-competent follicle								
<i>SFRP1</i>	3.05 ^c	0 ^d	Up 5'-TTCTAATGATTGGCAAGTCACG Low 5'-TGGCTGATTGAGATTCAGAGAC	NM_003012	Hs.713546	239	53	82
<i>HOMER1</i>	3.05 ^c	0	Up 5'-GGGAACAACCTATCTTCAGCAC Low 5'-ATTGCCTTTGAGCCATCTAAAC	NM_004272	Hs.591761	163	53	81
<i>GABPB1</i>	2.80 ^c	0	Up 5'-TCCCACAGAAATGATGAAAGTG Low 5'-TGATGGAAGGCACAGAATACAG	NM_005254	Hs.654350	223	53	81
Housekeeping genes								
<i>GAPDH</i>			Up 5'-ACCACAGTCCATGCCATCAC Low 5'-TCCACCACCCTGTTGCTGTA	NM_002046	Hs.544577	452	56	89
<i>ACTB</i>			Up 5'-CGTGACATTAAGGAGAAGCTGTGC Low 5'-CTCAGGAGGAGCAATGATCTTGAT	NM_001101	Hs.520640	375	58	88
<i>PPIA</i>			Up 5'-CAGCAGGCAGAGAAAATCCT Low 5'-GCATCTTTCGGGTTCTTTT	NM_007350	Hs.602085	421	59	83

^aHybridization performed in this experimentation; ^bHybridizations performed previously (Hamel et al., 2008); ^cFold change obtained by dye-swap hybridizations. ^dUndetectable; *EREG*, *Homo sapiens* epiregulin; *DPYSL3*, *Homo sapiens* dihydropyrimidinase-like 3; *PGR*, *Homo sapiens* progesterone receptor; *YWHAZ*, *Homo sapiens* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; *MARCKS*, *Homo sapiens* myristoylated alanine-rich protein kinase C substrate; *UGP2*, *Homo sapiens* UDP-glucose pyrophosphorylase 2; *SEMA3A*, *Homo sapiens* sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3A; *LRP8*, *Homo sapiens* low-density lipoprotein receptor-related protein 8, apolipoprotein e receptor; *PIR*, *Homo sapiens* pirin (iron-binding nuclear protein); *PHLDA1*, *Homo sapiens* pleckstrin homology-like domain, family A, member 1; *SFRP1*, *Homo sapiens* secreted frizzled-related protein 1; *HOMER1*, *Homo sapiens* Homer homolog 1 (*Drosophila*); *GABPB1*, *Homo sapiens* GA-binding protein transcription factor, beta subunit 1; *GAPDH*, *Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, *Homo sapiens* beta-actin; *PPIA*, *Homo sapiens* cyclophilin A.

embryologist. From patients recruited for the study (Table I), we selected patients who had 100% of the transferred embryos associated with successful pregnancies (pregnancy groups) and patients who had 100% of the transferred embryos associated with unsuccessful pregnancy (non-pregnancy group). Average numbers of oocytes recovered and fertilized were similar in both groups, but the average number of embryo transferred was higher in the non-pregnancy group ($P = 0.0023$).

Microarray hybridizations

Hybridizations with RNA from FCs were performed. A total of 62 transcripts had a \log_2 ratio (>2.0) consistent with preferential expression in the pregnancy group. Hybridization comparison was done with results already obtained previously from FCs from follicles leading to a pregnancy (Hamel et al., 2008) resulted in the identification of 31 common transcripts coding for 25 different genes. For transcripts preferentially expressed in the non-pregnancy group, we detected 54 transcripts with \log_2 ratios >2.0 .

Candidate gene selection

From the 25 candidate genes expressed preferentially in the pregnancy group (hybridization A) and in the two other hybridizations (hybridizations B performed previously Hamel et al., 2008), markers were selected and graded according to their known function, their number of occurrences in different libraries, their repetition in the same library and signal intensity. After selection and grading, 10 candidate genes were validated by qRT-PCR (Table II): epiregulin (EREG), dihydropyrimidinase-like 3 (DPYSL3), progesterone receptor (PGR), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ), myristoylated alanine-rich protein kinase C substrate (MARCKS), UDP-glucose pyrophosphorylase 2 (UGP2), semaphorin 3A (SEMA3A), low-density lipoprotein receptor-related protein (LRP8), pirin (PIR) and pleckstrin homology-like domain, family A, member 1 (PHLDA1).

From the 54 transcripts preferentially expressed in the non-pregnancy group in hybridization A (fold change >2.00), 24 transcripts were not expressed in hybridizations B previously done on FCs from follicles leading to a pregnancy. A total of 19 different

genes were found. Candidate markers were selected and graded according to their known function and signal intensity. After selection and grading, three candidate genes from the Non-Pregnancy Group were validated by qRT-PCR (Table II): secreted frizzled-related protein 1 (SFRP1), Homer homolog 1 (HOMER1) and GA-binding protein transcription factor β 1 (GABPB1).

Quantitative PCR

Quantitative RT-PCR was performed on all three pools of human FCs from each group (pregnancy and non-pregnancy groups; Table I). From the 10 candidate genes selected as indicators of pregnancy, two genes [UGP2 ($P = 0.0023$) and PHLDA1 ($P = 0.0461$)] showed a statistical difference between FCs of pregnancy and non-pregnancy groups (Fig. 1). UGP2 ($P = 0.0023$) had a higher gene expression in the pregnancy groups (Fig. 1). No differences in mRNA levels were observed between the two groups in the eight other genes selected (Table III). Expression of housekeeping genes ACTB, GAPDH and PPIA was similar ($P > 0.05$) in both groups (Table III).

From the three candidate genes selected as indicators of non-pregnancy, one gene [GABPB1 ($P = 0.0940$)] (Fig. 2) was not statistically different between the two groups, mainly due to larger variations in the levels measured, but could be considered as a potential indicator of follicular incompetence. No differences in the transcripts levels were observed for the two other genes selected (Fig. 2).

Discussion

Results presented in this study have identified UGP2 and PHLDA1 as potential follicular markers associated with embryo developmental competence resulting in a successful pregnancy. Furthermore, results obtained from GABPB1 showed a potential involvement of this follicular marker as an indicator of embryos resulting in an unsuccessful pregnancy. The identification of these markers may be added to the morphological evaluation of individual oocytes to improve the efficiency of predicting embryo competence and therefore increase the probability of a pregnancy with single embryo transfer.

In our previous studies, candidate genes were identified between populations of FCs from follicles leading to a pregnancy and cells

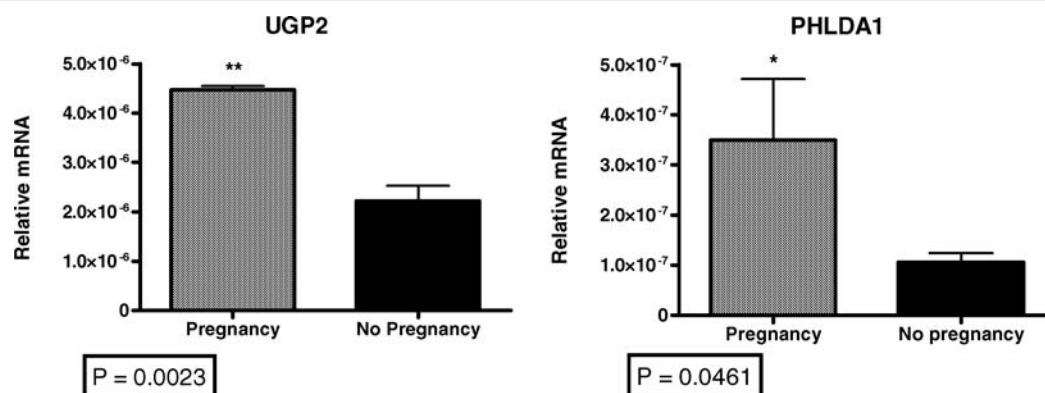


Figure 1 Gene markers associated with competent follicles. Quantification by qRT-PCR of mRNAs that showed differential expression ($P < 0.05$) in FCs from follicles associated or not with pregnancy. Results were analysed by *t*-test analysis and are presented as mean \pm SEM.

Table III Quantification by qRT–PCR of mRNAs that showed similar expression ($P > 0.05$) in FCs from follicles associated or not with pregnancy.

Gene	Mean pregnancy groups (Relative mRNA)	Pregnancy groups \pm SEM	Mean non-pregnancy groups (relative mRNA)	Non-pregnancy groups \pm SEM	Difference between means	P-value
SEMA3A	6.60E–07	1.88E–07	2.44E–07	1.49E–07	4.17E–07 \pm 2.40E–07	0.1564
YWHAZ	2.13E–07	2.26E–08	1.41E–07	4.54E–08	7.29E–08 \pm 5.07E–08	0.2242
PGR	6.68E–09	1.72E–09	5.01E–09	8.94E–10	1.66E–09 \pm 1.94E–09	0.4397
LRP8	3.91E–09	2.10E–09	3.79E–09	2.01E–09	1.22E–10 \pm 2.91E–09	0.4782
MARCKS	1.56E–07	1.35E–07	2.56E–07	1.51E–07	–1.00E–07 \pm 2.03E–07	0.6473
PIR	9.15E–08	1.79E–08	1.12E–07	4.41E–08	–2.00E–08 \pm 4.76E–08	0.6956
DPYSL3	7.83E–08	1.85E–08	8.84E–08	2.94E–08	–1.01E–08 \pm 3.47E–08	0.7850
EREG	5.80E–09	3.34E–09	5.59E–09	2.38E–09	2.07E–10 \pm 4.10E–09	0.9622
GAPDH	8.80E–07	1.16E–07	6.48E–07	9.86E–08	2.33E–07 \pm 1.52E–07	0.2008
PPIA	6.45E–06	2.79E–06	8.14E–06	3.22E–06	1.69E–06 \pm 4.26E–06	0.7116
ACTB	3.64E–06	5.17E–07	3.21E–06	1.03E–06	4.27E–07 \pm 1.16E–06	0.7308

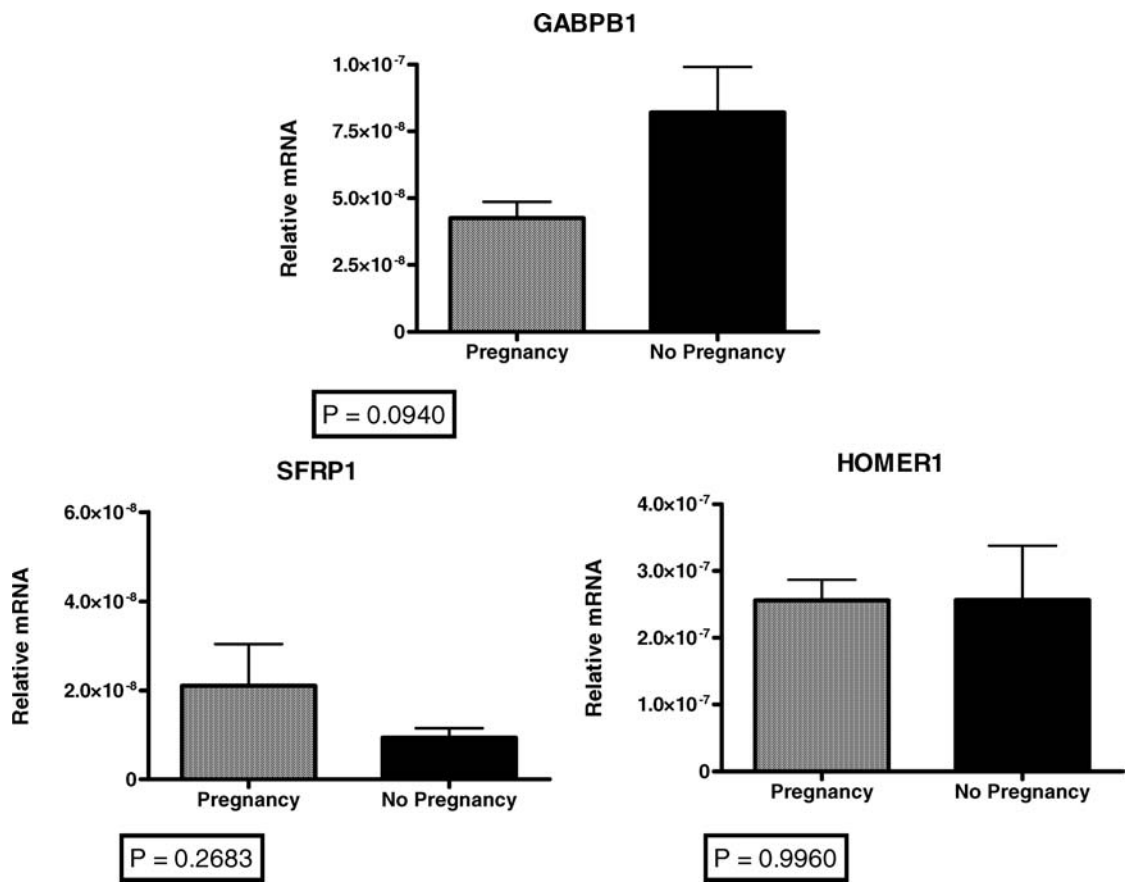


Figure 2 Gene markers associated with non-competent follicles. Quantification by qRT–PCR of mRNAs that showed a tendency ($P < 0.1$) or similar expression ($P > 0.1$) in FCs from follicles associated or not with pregnancy. Results were analysed by t -test analysis and are presented as mean \pm SEM.

from follicles associated with failed embryo development (Hamel *et al.*, 2008, 2010). However, from a clinical setting, only embryos of good morphological quality are transferred. For this reason, in this paper, candidate gene identification was pushed further to compare FCs from follicles associated with embryos that fulfilled all the morphological characteristics to be selected for embryo transfer,

but resulted in a successful or unsuccessful pregnancy outcome. In this study, we have different patients producing good embryos and not having the same outcome. The genes associated with this difference could be related to the overall response and for example illustrates changes associated with a lower uterine capacity to respond due to abnormal level of estradiol. It is therefore expected that the markers found before (Hamel et al., 2008, 2010) would be of a different kind since they are directly associated with subtle changes in the ovarian physiology and therefore creating a discrimination between early, normal and maybe late growing follicles. This difficulty is mitigated by using multiple patients in each group, by the technique of suppressive subtractive hybridization (SSH; see comments in Hamel et al. 2008) and by the validation using qRT-PCR. For clinical relevance, these optimal markers could successfully discriminate embryos achieving good morphological evaluation. The vast majority of differentially expressed transcripts between closely related populations of cells with slight physiological differences fall into the low-abundance transcripts (Evans et al., 2002; Cao et al., 2004). Sample processing, protocols used, hybridizations, detection systems and approaches applied to data analyses are some of the sources of variability causing biases in the identification of differentially expressed genes. To overcome the inherent biases of each approach and to improve the identification of differential expressed genes between these two similar populations, we used hybridizations and qRT-PCR validations. Microarrays, usually in combination with qRT-PCR, are recognized as the method of choice for gene expression analysis (Bosotti et al., 2007). Pools may to some extent mask some potential indicators of competence, but the ones that resisted the pooling dilution are even more likely to become valid indicators of useful differences. Further analysis of these genes on a much larger scale or clinical validation where the tissues of each patient from each follicle are currently studied.

It has been well established that the luteinization process starts before ovulation. In the context of ovarian stimulation in human IVF, high concentrations of LH/hCG can cause early luteinization of granulosa cells (Hillier, 1994). In this study, genes expressed in FCs associated with pregnancy could be related to the early stage of corpus luteum formation. Therefore, competent follicles could be those which have rapidly started the luteinization process after LH/hCG surge.

UGP2 is an enzyme involved in glycogenesis (Kleczkowski et al., 2004). UDP-glucose pyrophosphorylase activity is increased after LH stimulation in human oocytes (Yazigi et al., 1993). The catalytic product of UGP enzymes, UDP-glucose, can be used by granulosa cells for the synthesis of glycosaminoglycans like hyaluronan (HA; Magee et al., 2001). It has been reported that the addition of HA to embryo culture medium increased pregnancy rates in bovine IVF (Block et al., 2009). HA expressed in mural granulosa cell layers has a potential role in cell locomotion (Turley, 1992), and plays an important role in preventing oocyte degeneration (Sato et al., 1987). During ovulation, the extracellular matrix of the ovarian surface is degraded to allow release of the oocyte. Therefore, LH/hCG stimulation could induce a rapid change in gene expression involved in matrix remodeling for the luteinization process.

PHLDA1 is involved in a well-controlled anti-apoptotic process to maintain tissue homeostasis and prevent accumulation of damaged cells. Down-regulation of PHLDA1 expression is associated with the

progression of cancer by deregulation of cell growth and increased cell sensitivity to apoptosis (Neef et al., 2002; Marchiori et al., 2008). In this way, the expression of PHLDA1 in FCs associated with fully developmental oocyte could be related to the early luteinization process induced by LH/hCG surge in granulosa cells to prevent apoptosis.

Interestingly, GABPB1 showed a tendency to be expressed in FCs related to oocytes associated with no pregnancy. Using a custom-made cDNA microarray from subtracted libraries enriched with transcripts of FCs from follicles associated with pregnancy, this discovery was unexpected. The stringency of SSH can be modified by changing the ratio of driver/tester (Desai, 2000). Increasing this ratio allows preferential enrichment of genes that are highly up-regulated in the tester compared with the driver in the subtracted library. However, for a close, similar population of cells such as those used in our study, cDNAs with smaller differences in expression are lost during SSH. Conversely, decreasing the ratio of driver/tester allows the identification of genes that are slightly up-regulated in tester, but also increases the occurrence of false positive clones. Because we wanted to have a large screen of potential candidate genes and since further hybridizations on custom-made array and qRT-PCR can eliminate the majority of false positive clones, we did not change the recommended protocol's ratio. Therefore, the custom-made granulosa chip can be a useful tool to discriminate genes with potential involvement in follicles from embryos associated with unsuccessful pregnancy.

GABP is a member of the ETS transcription factor family that stimulate the transcription of genes that control the cell cycle, protein synthesis and apoptosis (Rosmarin et al., 2004). GABP is also involved in cellular signalling pathways by regulating hormones and transmembrane receptors (Rosmarin et al., 2004). During the mid to late stages of follicular development, granulosa cells are rapidly dividing when they acquire FSH responsiveness (Robker and Richards, 1998) and prepare the follicle for the luteinization process (Yamada et al., 1999). In accordance with our results, it has been reported that GABP seems to be expressed in granulosa cells before ovulation (MacLean et al., 2005), supporting the possible involvement of GABP in the granulosa luteinization process. Therefore, FCs with higher mRNA expression of GABPB1 could indicate that their associated follicles are well engaged in the apoptosis process and therefore lead to lower probabilities of successful pregnancy.

There is growing evidence that FCs can reflect the quality of the oocyte obtained at ovulation (for review: Li et al., 2008). The LH/hCG surge triggers the expression of multiple genes in FCs, which are involved in many diverse pathways that are essential to achieve ovulation finality. Strong evidence indicates that genes associated with the granulosa luteinization process are promising indicators of follicular ability to produce an embryo with full capacity to result in a pregnancy. The luteinization process seems to be a crucial step during follicular development to produce a fully healthy oocyte.

Authors' roles

M.H. designed the study, performed experiments and wrote the paper. I.D., C.R. helped with analysis. M.-C.L. provided follicular cells, helped with analysis. A.L. provided follicular cells. M.A.S. designed the study.

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