New candidate genes to predict pregnancy outcome in single embryo transfer cycles when using cumulus cell gene expression

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Objective: To relate the gene expression in cumulus cells surrounding an oocyte to the potential of the oocyte, as evaluated by the embryo morphology (days 3 and 5) and pregnancy obtained in single-embryo transfer cycles.
Design: Retrospective analysis of individual human cumulus complexes using quantitative real-time polymerase chain reaction for 11 genes.
Setting: University hospital IVF center.
Patient(s): Thirty-three intracytoplasmic sperm injection patients, of which 16 were pregnant (4 biochemical and 12 live birth).
Intervention(s): Gene expression analysis in human cumulus complexes collected individually at pickup, allowing a correlation with the outcome of the corresponding oocyte. Multiparametric models were built for embryo morphology parameters and pregnancy prediction to find the most predictive genes.
Main Outcome Measure(s): Gene expression profile of 99 cumulus complexes for 11 genes.
Result(s): For embryo morphology prediction, TRPM7, ITPKA, STC2, CYP11A1, and HSD3B1 were often retained as informative. Models for pregnancy—biochemical or live birth—complemented or not with patient and cycle characteristics, always retained EFNB2 and CAMK1D together with STC1 or STC2. Positive and negative predictive values of the live birth models were >85%.
Conclusion(s): EFNB2 and CAMK1D are promising genes that could help to choose the embryo to transfer with the highest chance of a pregnancy. (Fertil Steril® 2012;98:432–9. ©2012 by American Society for Reproductive Medicine.)
Key Words: Human cumulus cell, gene expression, oocyte quality, pregnancy, live birth

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Multiple pregnancies still remain a major concern in assisted reproductive technology (ART) when replacing more than one embryo to increase the chance for pregnancy per cycle. Single-embryo transfers (SETs) avoid this risk. To improve the pregnancy chance per cycle when performing SET, more criteria to select the embryo with the highest implantation potential may be implemented. For several years now, cumulus cell (CC) gene expression has been investigated as a tool to predict the quality of the oocytes (1, 2) along with the morphologic criteria of the embryos. CC analysis is an obvious choice for a noninvasive analysis, because CCs are in direct contact with the oocyte and voided during the intracytoplasmic sperm injection (ICSI) procedure.

In a previous study, models were designed to predict embryo morphology features and clinical pregnancy with the use of gene expression in CCs that had been removed shortly before ICSI (3). The genes analyzed originated

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from previous experiments using Affymetrix arrays resulting in 500 genes related to pregnancy (unpublished data). Out of eight tested genes with quantitative polymerase chain reaction (PCR) (3), the four most informative genes were transient receptor potential cation channel, subfamily M, member 7 (TRPM7) and inositol-trisphosphate 3-kinase A (ITPKA) for better cleavage-stage embryo prediction and syndecan-4 (SDC4) and versican (VCAN) for pregnancy prediction. Those four genes together with seven newly chosen genes (also originating from the same array data) were retained to analyze cumulus complexes in the present study. The additional seven genes were: stanniocalcin-1 (STC1), stanniocalcin-2 (STC2), parathyroid hormone–like hormone (PTHLH), ephrin-B2 (EFNB2), cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), hydroxy-delta5-steroid dehydrogenase, 3beta- and steroid delta-isomerase 1 (HSD3B1), and calcium/calmodulin-dependent protein kinase 1D (CAMK1D).

Stanniocalcin is a secreted glycoprotein hormone that was first described in bony fish, where it prevents hypercalcemia (4). In cultured rat granulosa cells, STC1 and STC2 decreased the FSH-induced progesterone production which was paired with a decrease in CYP11A1 and HSD3B (5, 6). In swine granulosa cell cultures, STC1 production increased when the granulosa cells were put in hypoxic conditions (7). The close association with steroidogenesis of both STCs led us to investigate CYP11A1 and HSD3B1 in the same sample set. CYP11A1 is responsible for the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of the steroid hormones (8). HSD3B transforms pregnenolone into progesterone (9). HSD3B1 has previously been described as a positive marker when comparing the expression of human follicular cells from an oocyte resulting in pregnancy with those from an oocyte giving an embryo arrested in development (10).

PTHLH is known to be important during the development of several organs, including the mammary glands (11). PTHLH is involved in lactation possibly by regulating the mobilization and transfer of calcium to the milk (12). It also plays a role in the development of hypercalcemia in patients with small cell carcinomas of the ovary (13). In porcine granulosa cell culture it was demonstrated that transforming growth factor (TGF) β1 could increase the PTHLH concentrations (14), and TGF-β1 family members such as GDF9 and BMP15 are known to help regulate CC function (15).

EFNB2 is a transmembrane protein that belongs to the largest subfamily of receptor protein–tyrosine kinases and has been implicated in mediating developmental events, especially in the nervous system and in erythropoiesis (16). EFNB2 expression was described in human granulosa cells mainly during luteinization (17). The EFNB2 receptor, EPHB2, was shown to increase in mice granulosa cells when injecting pregnant mare serum gonadotropin in wild-type mice but not in estrogen beta-receptor–null mice. This suggests a regulation of EPHB2 expression by FSH and the estrogen beta-receptor (18).

CAMK1D encodes a member of the Ca-/calmodulin–dependent protein kinase 1 subfamily of serine/threonine kinases. CAMK1D itself has never been reported in the ovary. Other members of the gene family have, however, been related to ovarian events such as oogenesis (CAMK1, CAMK2A, and CAMKG), folliculogenesis (CAMK4), ovulation (CAMK4 in granulosa (GC) and theca cells (TC)), and corpus luteum formation where it may serve as a Ca^{2+}-dependent effector mechanism to maintain basal CYP11A gene expression (CAMK4) (19–21).

Genes coding for diverse pathways in the cell (calcium, steroidogenesis, extracellular matrix formation, and TGF-β–related pathways) were chosen to enhance the predictive power of the models described hereafter and to avoid collinearity.

First, we evaluated the inter- and intrapatient variation for the 11 genes in CCs. Second, we explored the possibility to predict the most relevant morphologic features of day 3 and day 5 embryos with the 11 genes complemented with patient and cycle characteristics with the use of stepwise multiple regression analysis.

In a third multiparametric approach, models only allowing gene expression values were determined first for biochemical pregnancy (positive hCG value) and second only for live birth pregnancy. In the next step, patient and cycle characteristics were introduced in the models if they improved them significantly.

Finally, results were validated by randomly splitting the patients into two halves, with the first group building the model for pregnancy and the second group testing the predictive power of the polynomials.

MATERIALS AND METHODS

Patient Population

This study was approved by the Ethical Committee of Universitair Ziekenhuis Brussel, and patient consents were obtained. Consecutive patients were chosen based on the stimulation protocol and transfer strategy (SET). Patients underwent controlled ovarian hyperstimulation by administration of GnRH antagonist combined with recombinant FSH (Gonal-f, Serono [n = 5]; or Puregon, Shering-Plough [n = 28]). Follicular development was monitored by vaginal ultrasound. The endocrine profile was evaluated by analysis of serum E_2, P, FSH, and LH. A dose of 10,000 IU hCG was administered to induce final follicular maturation when at least three follicles 17 mm in diameter were observed by transvaginal ultrasound. Cumulus oocyte complexes (COCs) were aspirated 36 hours later. ICSI was performed as described previously (22), and embryo culture was performed in sequential media of Vitrolife G7. Out of the 33 patients, 13 had an embryo transfer on day 3 and 20 had a blastocyst transfer on day 5.

Infertility causes were: male factor (n = 18), female factor (ovulation disorder [n = 1], endometriosis [n = 1], and myomatosis [n = 1]), a combination of male and female factors (tubal pathology [n = 2] and PCO [n = 2]), and idiopathic (n = 8).

Out of the 33 patients, 16 were pregnant after SET (four were biochemical pregnancies, and 12 delivered a live born).

Collection of CCs

COCs were retrieved 36 hours after hCG and collection of the CCs was carried out as described earlier (3). Briefly: After washing the COCs at collection, oocytes were denuded in

individual 40-μL droplets of HTF-SSS containing 80 IU/mL Cumulase (MediCult) for a maximum of 30 seconds and washed sequentially in two droplets without enzyme. Immediately after dissociation from the oocyte, the CCs were plunged into liquid nitrogen. From this point onwards, oocytes were kept individually throughout the culture period, allowing correlation with their respective CCs.

From the 16 pregnant patients, the CCs of all fertilized oocytes were analyzed (n = 82). From the 17 nonconceiving patients, only CCs of the fertilized oocytes that were transferred were analyzed (n = 17).

Morphology Parameters Considered for Oocyte Developmental Competence

The following parameters reflecting oocyte developmental competence were considered: 1) ≥7 cells on day 3, reflecting developmental speed; 2) low fragmentation rate on day 3, i.e., ≤10% fragmentation; 3) top embryos on day 3, having ≥7 blastomeres with ≤10% fragmentation (23); 4) good embryos on day 3, having ≥7 blastomeres and ≤20% fragmentation; 5) fast-developing blastocysts on day 5, i.e., BL4–5 (24) regardless of the inner cell mass (ICM) and trophectoderm (TE) scores compared with BL1–2; 6) top blastocysts on day 5, i.e., BL3–5 and AA or AB ICM/TE score with ≤10% fragmentation; and 7) good blastocysts on day 5, i.e., BL1–2 or BL3–5 with minimum BB ICM/TE score and ≤20% fragmentation.

RNA Extraction and cDNA Synthesis

Total RNA was extracted as described earlier (25). In brief, the extraction was performed with the use of the RNeasy Micro Kit (Qiagen) on the Qiacube (Qiagen) using the RNeasy DNase-digestion extraction protocol, where 5 ng/μL poly(dA) (Roche Applied Science) was added before extraction. After extraction, a second DNase treatment (RQ1 RNase-Free DNase; Promega) was performed according to the manufacturer's protocol in a 22-μL reaction volume. This RNA was diluted 1:2 at the end and stored at −80°C.

Reverse transcription was performed on 40 μL RNA with the use of the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's protocol with a reaction volume of 60 μL subsequently diluted to 100 μL. Two negative control samples were generated by omitting the reverse transcriptase enzyme or the RNA in the RT reaction. All cDNA was stored at −80°C until PCR analysis.

Real-Time PCR

Primer sequences for TRPM7, ITPKA, VCAN, and SDC4 are listed elsewhere (3, 25). For CAMK1D a Taqman gene expression assay Hs00220668_m1 (Applied Biosystems) was used following the manufacturer's instructions, adapted to 15 μL reaction volume. Primers for STC1, STC2, EFNB2, PTHLH, CYP11A1, and HSD3B1 were designed using the Universal Probe Library software (Roche Diagnostics, Roche Applied Science) and were chosen to be intron spanning (Supplemental Table 1, available online at www.fertstert.org). The geometric mean of the beta-2-microglobulin (B2M) and ubiquitin C (UBC) expression was considered to be the endogenous normalization factor (3). All PCRs, except for CAMK1D, were performed in 15-μL reactions of LC480 Sybr Green I Master (Roche Diagnostics) with 2 μL cDNA and 0.6 μmol/L primer concentration for the genes of interest and 0.3 μmol/L for the endogenous controls. Activation was done at 95°C for 10 minutes. Cycling conditions were: 40–50 cycles of 10 seconds at 95°C and 30 seconds at 60°C. All samples were run in duplicate or triplicate and quantified with the use of a standard curve generated by a 6-log dilution of a synthetic oligonucleotide equal to the amplicon. On each plate, a PCR no-template control was included. To assess the specificity of amplification, a melting curve was carried out on all samples and sequenced once, except for CAMK1D. All negative control samples were as expected, and all results mentioned later are the values normalized for the mean concentrations of B2M and UBC for that sample.

Statistical Analysis

Gene correlations. Correlations (Pearson) between the different genes were performed for the 99 cumulus cell samples. To represent 41% of the total variability of the multivariate dataset, a biplot was drawn along the first and second principal components.

Between- and within-patient expression variation. Between- and within-patient variations of the hierarchic model were calculated using a linear mixed model with patient as a random factor, where the total variability consisted of a within-patient variability part and a between-patient variability part.

Embryo development. Models predicting embryo development were built by stepwise multiple regression analysis as described previously (3). On all expression results of all 2PN oocytes (82 from pregnant patients and 17 from nonpregnant patients), a generalized linear regression model ($y = a + bx + cz + ds + et + fu + gv + hw$) using a logit-link was built with the outcome parameters (listed in a preceding section and Table 1) as response variables. X, z, and s were expression values of different genes supplemented with patient and treatment-related variables: t, u, v, w with b–h as their respective indexes, and a as the intercept. The patient and cycle characteristics were used to compensate for the between-patient variation where needed. In the first step, the expression values were added progressively if they improved the model. In a second step, the best-fitting patient or cycle characteristics were added, and this was repeated until addition did not further improve the model. Finally, a backward regression step was performed excluding nonessential variables from the equation. All steps were evaluated based on the overall value of the model together with the type III *P* values. A type III *P* value of .3 was used as the final criterion to put and keep variables.

Pregnancy. For all pregnancy analysis only the transferred oocytes were considered. A two-tailed *t* test was used to compare expression values of cumulus complexes of oocytes resulting in pregnancy or not (n = 16 pregnant; n = 17 nonpregnant). All data was log transformed to obtain normal distribution, and a Bonferroni correction was applied,

TABLE 1

Patient, cycle, and embryo development characteristics.

	Pregnant			Nonpregnant			
Variable	**Mean**	**SD**	**n**	**Mean**	**SD**	**n**	***t* test**
Age (y)	31	4	16	30	3	17	ns
Body mass index (kg/m^2)	23	5	15	24	5	17	ns
Days of stimulation (n)	10	2	16	9	2	17	ns
Gonadotropin dose (U/d)	171	38	16	178	29	17	ns
FSH[a] (U/L)	12	5	13	12	3	14	ns
LH[a] (U/L)	1.00	0.56	10	3.26	4.35	13	ns
Relative E_2[b] (ng/L)	130	59	13	186	81	13	ns
P[a] (μg/L)	1.12	0.47	13	1.03	0.58	14	ns
COCs retrieved at pickup (n)	10	5	16	12	7	17	ns
Ovarian response[c] (n)	6	3	16	7	4	17	ns
Oocyte maturity[d] (%)	82	17	16	78	21	17	ns
2PN[e] (%)	77	26	16	74	28	17	ns
$\geq$7 cells day 3[f] (%)	77	24	16	77	28	17	ns
Low fragmentation[g] (%)	61	39	16	83	28	17	ns
Good-quality embryos[h] (%)	52	27	16	52	32	17	ns

Note: ns = P>.05.
[a] Serum values as measured on day of hCG.
[b] E_2/COCs retrieved.
[c] COCs retrieved/gonadotropin dose × 100.
[d] Metaphase II oocytes/COCs retrieved.
[e] 2-pronuclear/intact oocytes after intracytoplasmic sperm injection.
[f] Embryos with ≥7 cells on day 3/2PN.
[g] Embryos with <10% fragmentation on day3/2PN.
[h] Embryos on day 3 with <10% fragmentation and at least 7 cells/2PN.

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considering only P values < .0045, resulting in a global type I error rate of the analysis of .05.

In a second analysis for pregnancy, two models were built up the same way as for the embryo development. The difference here was that in the first model only the gene expression values were allowed. In the second model, parameters from Table 1 were allowed if they improved the model.

The third analysis was a proof of concept by randomly splitting up the patients into two groups. A pregnancy model was built up with only ten of the pregnant patients and ten of the nonpregnant patients randomly allocated by a computer. The model calculated from those 20 patients was used to predict the pregnancy outcome of the six pregnant and seven nonpregnant patients not included in the model.

Positive predictive value (PPV = true positive/[true positive + false negative]), negative predictive value (NPV = true negative/[true negative + false positive]), and accuracy ([true positive + true negative]/[true positive + false positive + false negative + true negative]) were calculated for each pregnancy model. Graphpad software was used for the correlations and the two-tailed *t* test. Multiple regression statistics were performed with the use of S + 7.0 for Linux (Tibco).

RESULTS

Patient Population

Table 1 presents the patient and cycle characteristics of pregnant and nonpregnant patients. No significant differences in patient, cycle, and embryo development characteristics were observed between the pregnant and nonpregnant patient groups.

Expression Correlations

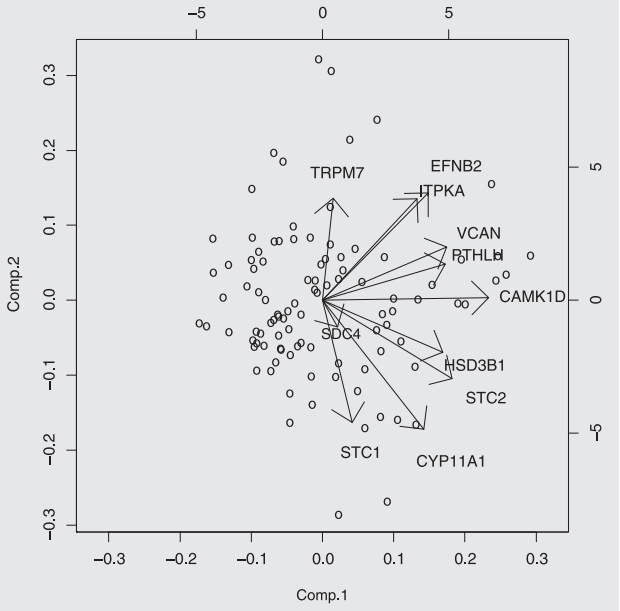
Only significant (P< .001) correlations between the different genes were considered and presented in Supplemental Table 2 (available online at www.fertstert.org). SDC4 and TRPM7 had no strong correlations with the other genes. CAMK1D on the other hand was the most central gene by having strong correlations with six genes, whereas the other genes correlated with a maximum of three genes (STC2, EFNB2, CYP11A1, HSD3B1, and VCAN). It is therefore not surprising to find CAMK1D as the most central gene in the biplot (Fig. 1). Two groups of genes that can be found in the biplot are the steroid-related genes (STC1, STC2, CYP11A1, and HSD3B1) and the calcium-related genes (TRPM7, ITPKA, PTHLH, and CAMK1D). EFNB2 and VCAN are closer to the calcium-related genes and SDC4 can be found in between the steroid-related genes. EFNB2 correlated with CAMK1D, and they both had in common the correlations with ITPKA and VCAN. The other three genes with which CAMK1D correlated are steroid-related genes STC2, CYP11A1 and HSD3B1. All genes that correlated with CAMK1D also correlated with two other genes, except for ITPKA (only one extra). CYP11A1 correlated with HSD3B1 and STC2, as might be expected, and STC2 with STC1. PTHLH had only two correlations, i.e., HSD3B1 and VCAN. All correlations between the different genes were positive.

Intra- and Interpatient Expression Variation

Results for the 11 genes can be found in Supplemental Table 3 (available online at www.fertstert.org).

STC1 and ITPKA had the highest intrapatient variation, and both STCs together with PTHLH and ITPKA had the highest

FIGURE 1



Biplot of the 11 genes along the first and second component. Dots represent each cumulus cell (CC) sample (n = 99). The multivariate scatter of the whole dataset is condensed in the first two principal components, which represent 41% of the total variation. The lower and left axes are the coordinates for the arrows (indicating the loadings of the gene expressions) and the upper and right axes show the coordinates for the individual points (indicating the scores of the individual points). The closer the dots, the more similar the gene expression pattern is for these CC samples according to both principal components. The closer the arrows are to each other, the more the gene expressions are correlated.

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interpatient variation. The genes with the smallest intrapatient variation were CYP11A1, HSD3B1, VCAN, CAMK1D, and EFNB2 and those with the smallest interpatient variation were VCAN and TRPM7. The seven newly described genes had a higher interpatient variation than intrapatient variation, except for CAMK1D where both variations were quite similar. For the seven new genes, smaller interpatient variation related to smaller intrapatient variation. From the four genes taken over from the previous study, TRPM7 had lower interpatient variation than intrapatient variation. The three remaining genes had quite similar inter- and intrapatient variations.

Models Predictive for Embryo Morphology

Four models for morphology could be built (Table 2), i.e., low fragmentation rate on day 3, top embryos on day 3, fast-developing blastocysts, and good blastocyst morphology on day 5. Models for the number of cells on day 3, good embryos on day 3, and top blastocysts on day 5 could not be built, because the number of samples in the positive or the negative group was too low (<20% of the total number).

Models Predictive for Pregnancy

In a first step, a *t* test was performed between the CC samples from oocytes that resulted in pregnancy (at least biochemical)

TABLE 2

Schematic overview of the strongest morphology and pregnancy models.

	n (n)[a]	TRPM7	ITPKA	STC1	STC2	EFNB2	CYP11A1	HSD3B1	CAMK1D	Prog	Rel. E_2	Days of stim.	*P* model	PPV	NPV	Acc.
Low fragmentation on day 3	99 (68)		x		x		x				x		.0007			
Top embryos on day 3	99 (56)	x								x		x	.0026			
Fast developing blastocyst	27 (17)	x			x			x					.0305			
Good blastocysts on day 5	62 (22)						x			x		x	.0012			
Biochemical pregnancy																
Only genes	33 (16)			x		x			x				.00002	80	78	79
All factors allowed	26 (13)			x		x			x		x		.000068	92	92	92
Proof of concept	20 (10)			x		x			x				.02806	71[b]	83	77
Live birth																
Only genes	29 (12)				x	x			x				.00276	91	89	90
All factors allowed	29 (12)				x	x			x			x	.01048	91	89	90

Note: Only genes and factors that were at least retained once are listed. "x" means that the factor significantly improved the model. NPV = negative predictive value; PPV = positive predictive value; Rel. E_2 = E_2 at day of hCG/no. of COCs retrieved; stim. = stimulation.

[a] n = total number of samples used to build the model; (n) = number of positive samples for the criterion.

[b] PPV, NPV, and accuracy (Acc.) were calculated on the remaining 13 (6) patients.

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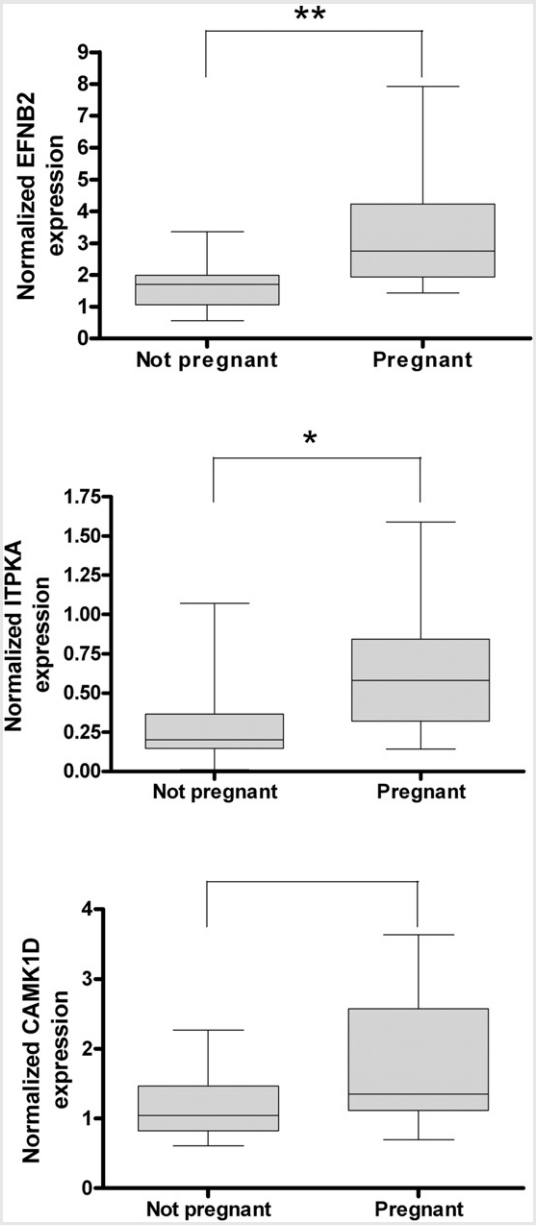
FIGURE 2



Not pregnant (n = 17) versus pregnant (n = 16) *t* tests on log-transformed expression values: *$P<.0045$; **$P<.001$. Boxes and whiskers represent the total range and quartiles with a line at the median.

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after transfer and those that did not. ITPKA and EFNB2 were significantly up-regulated in the pregnant group (Fig. 2), and CAMK1D showed the same trend.

In a second step, multiparametric models were built up, first only based on gene expression, second also using patient, cycle, and embryo characteristics. Both models were once built up for all pregnant patients, including biochemical pregnancies (n = 4) and once only for the patients that gave live birth (n = 12). Mathematical models can be found in Supplemental Table 4 (available online at www.fertstert.org). EFNB2 and CAMK1D were retained in all pregnancy models. STC1 and STC2 alternated between, respectively, the biochemical + live birth and the live birth models. PPV and NPV varied from 78% to 92% for the different models and the accuracies from 79% to 92% (Table 2).

In a third step, a model was calculated using only part of the dataset (ten pregnant and ten nonpregnant patients randomly selected with the computer). Also here EFNB2, CAMK1D, and STC1 were retained for the model and the obtained PPV and NPV were 71% and 83% for 6 pregnant and 7 nonpregnant patients independent of the model. This concept was not repeated for the live birth pregnancies, because the number was too low.

DISCUSSION

We analyzed known and newly described genes in cumulus cells as possible oocyte quality markers in ART.

Although patient and cycle characteristics were similar in both pregnant and nonpregnant patients, there is still a rather large interpatient variation, mostly higher than intrapatient variation. Genes with a higher interpatient variation might require the use of a multiparametric approach allowing correction for patient and/or cycle characteristics. When comparing the values for the four genes that were also analyzed in a previous study (3), we saw that SDC4 and VCAN had similar variations both within and between patients, whereas TRPM7 had slightly increased inter- and intrapatient variations in the present study although the interpatient variation remained small. ITPKA, in contrast, had a smaller intrapatient variation in the present study, but it also remained among the largest detected, as reported previously.

In the present study, multiparametric regression models predicting development of embryos were significant and included at least one gene. Four genes analyzed in the present study in relation to morphology were also analyzed in a previous study (3). SDC4 and VCAN was not retained in the present morphology models, whereas ITPKA and TRPM7 proved to be among the strongest predictors also in the present gene set. TRPM7 is proposed to be an important predictor for top-morphology embryos on day 3 of culture in both studies. It should be noted that different culture media used in the two studies may result in different embryo development (26). This can in part explain the fact that we did not find exactly the same morphology models in the present dataset as in our previous study. These findings might indicate that when creating models for morphology outcome, the culture conditions should be taken into account and can influence the relations found between morphology outcome and gene expression levels of the CCs. Next to this it is also possible that the newly described genes are more potent than TRPM7, ITPKA, SDC4, and VCAN in some of the morphology models. For example, CYP11A1 improved the *P* value of the model for blastocyst quality on day 5 and ruled out SDC4 which was previously included in that model. However, the biplot shows that both genes are closely related. VCAN had a strong correlation with EFNB2 and would not be retained in a model if EFNB2 is present, because VCAN would not be able to improve the model because it would not explain different variations than EFNB2.

ITPKA and EFNB2 expressions were statistically higher in the CCs of oocytes giving pregnancy, and CAMK1D showed the same trend. In the multiparametric analysis, EFNB2 and CAMK1D were present in all models for pregnancy prediction together with STC1 or STC2. When comparing the present results with the previous study, SDC4, VCAN, and TRPM7 were no longer found to be significantly different in the *t* tests for pregnant versus nonpregnant, nor were SDC4 and VCAN retained in the multiparameteric models built for pregnancy prediction. A possible explanation for this is that, as mentioned above, a different culture medium was used from oocyte pickup to denudation. So far, no studies in human CCs have been performed to assess the influence of the type of culture medium for COCs before denudation on gene expression levels.

We also compared the characteristics of the pregnant and nonpregnant patients from the two studies (data not shown). The only variable that was significantly higher in the pregnant group of the previous study was the endogenous LH on the day of hCG. This could explain why LH-responsive genes, such as SDC4, VCAN, and TRPM7 (27, unpublished mouse data), had lower basal gene expression levels in the pregnant group of the present study. This could result in no differences at all between the pregnant and the nonpregnant groups in this dataset in CCs after hCG exposure. The loss of SDC4 and VCAN in the multiparametric models could be due to the fact that in the previous study we used the data from patients with two different stimulation protocols (agonist rhMG and antagonist rFSH) to build up the pregnancy models. It has been shown in different studies that gonadotropin molecules influence the granulosa cell gene expression (25, 28–30). But most likely, EFNB2 and CAMK1D are stronger predictors of pregnancy in the current patient population. To support this idea, models were built with only ten pregnant and ten nonpregnant patients from the investigated group. Even in this smaller subset of samples, the same three genes, EFNB2 and CAMK1D together with STC1, were again retained and resulted in a significant model. Using the model on the other part of the samples gave a PPV of 71%, an NPV of 83% (six pregnant, seven not pregnant), and an accuracy of 77%. This analysis indicates that CC gene analysis could be used prospectively for pregnancy prediction. Until now, no studies have been published using cumulus genes as predictors of pregnancy with prospective data in an SET setting.

When allowing patient- and cycle-related factors in the pregnancy model, only the PPV and the NPV for the biochemical + live birth model was increased. The PPV and NPV for live birth were already high when building the model on the basis of only three genes. This might result from the fact that both genes had a low interpatient variation. Embryo morphology parameters, such as numbers of cells and percentage fragmentation, were never retained in the predictive model, indicating that gene expressions were more strongly correlated with pregnancy, as was found in the earlier study (3).

Models predictive for morphology and pregnancy did not contain the same genes (except for STC2), confirming the findings by other groups (31, 32). It is known that current morphologic parameters are not strongly predictive for pregnancy (33). In a first prospective trial it might imply that the gene expression should still be combined with the current morphologic criteria. In such a setting morphology criteria can be used as exclusion criteria, as was demonstrated in a study by Meseguer et al. (34).

There is currently no clear explanation why EFNB2, CAMK1D, and STC1 are informative in pregnancy prediction. Some speculations can be made from the literature. Lanner et al. (35) showed that vascular endothelial growth factor (VEGF) increased the expression of EFNB2 in endothelial cells. The higher EFNB2 in the CCs might thus be the reflection of a higher VEGF content in the follicles and better vascularization of the follicles. This is supported by a study by Monteleone et al. 2008 (36) showing that a higher VEGF content in follicular fluid correlated with the grade of perifollicular vascularity and that a higher vascularization resulted in better fertilization rates, better embryos, and a higher pregnancy rate. STC1 was not significantly different between the two patient groups, but tended to be lower in the pregnant patients. A possible reason for higher STC1 expression in the nonpregnant group is that CCs from the oocytes not resulting in pregnancy might have been subjected to more stress by hypoxia. Basini et al. (7) showed that hypoxic conditions could increase STC1 expression. This can be linked to the proposed explanation of EFNB2 that less vascularization can lead to more hypoxic conditions and suboptimal oocytes. For CAMK1D no ovarian function has yet been reported. When looking at the correlation analysis, CAMK1D is a central gene. It had strong correlations with six of our selected genes. The correlation with ITPKA might be explained by the direct link of both genes to calcium-related pathways. In the present study, CAMK1D also had strong correlations with EFNB2 and VCAN. The latter gene is important for extracellular matrix formation after ovulation. Most strikingly, CAMK1D was correlated with three out of four steroid-related genes, indicating that CAMK1D is also involved upstream or downstream of this pathway. This may confirm the findings that a family member of CAMK1D, CAMK4, can increase the CYP11A1 expression in granulosa-luteal cells (20). The role of the three most promising genes in pregnancy prediction might be studied in an in vitro model (37), allowing observation and regulation during the follicular growth and post-LH phases.

In conclusion, the main challenge in SET cycles consists in increasing the chance of pregnancy per transfer cycle. Several research groups have tried to demonstrate that CC gene expression is a good way to achieve this goal. The present study identified new candidate genes to predict pregnancy outcome between a group of oocytes from different patients in SET cycles. CAMK1D, EFNB2, and STC1 seem to be strong genes to help choose the best embryo to transfer. Those genes should be tested, not only in a larger and more diverse SET patient population, but also in a setup where intrapatient variation can be analyzed, e.g., by comparing cumulus complexes from oocytes that did not result in pregnancy in a first cycle but for which a subsequent frozen embryo transfer cycle resulted in a live birth.

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SUPPLEMENTAL TABLE 1

Primer sequences (5′→3′).

Gene ID	Primer sequences	Accession no.
STC1	aggcggagcagaatgactc gttgaggcaacgaaccactt	NM_003155
STC2	tacctcaagcacgacctgtg gaggtccacgtagggttcg	NM_003714
CYP11A1	caccttcaccatgtccagaa ataaaccgactccacgttgc	NM_000781
HSD3B1	tcatgaatgtcaatgtgaaaggt ggcacactagcttggacaca	NM_000862
EFNB2	tctttggagggcctggat ccagcagaacttgcatcttg	NM_004093
PTHLH	ctcggtggagggtctcag tggatggacttccccttgt	NM_098966
CAMK1D	Hs00220668_m1	NM_153498

Wathlet. Cumulus gene expression and pregnancy. Fertil Steril 2012.

SUPPLEMENTAL TABLE 2

Pearson correlations between different gene expression values (n = 99).

	SDC4	TRPM7	ITPKA	STC1	STC2	PTHLH	EFNB2	CYP11A1	HSD3B1	CAMK1D	VCAN
SDC4											
TRPM7											
ITPKA							***			***	
STC1					***						
STC2								***		***	
PTHLH									***		***
EFNB2										***	***
CYP11A1									***	***	
HSD3B1										***	
CAMK1D											***
VCAN											

*** $P < .001$.

Wathlet. Cumulus gene expression and pregnancy. Fertil Steril 2012.

SUPPLEMENTAL TABLE 3

Intra- and interpatient variation.

	Intrapatient variation		Interpatient variation	
Gene	**Estimate**	**95% CI**	**Estimate**	**95% CI**
STC1	0.6	0.50–0.71	0.87	0.61–1.24
STC2	0.44	0.37–0.52	0.75	0.56–1.01
PTHLH	0.43	0.37–0.51	0.65	0.48–0.89
EFNB2	0.32	0.27–0.38	0.45	0.32–0.63
CYP11A1	0.24	0.21–0.29	0.41	0.30–0.56
HSD3B1	0.27	0.23–0.32	0.42	0.31–0.58
CAMK1D	0.31	0.26–0.37	0.38	0.27–0.53
SDC4	0.38	0.33–0.46	0.41	0.28–0.60
TRPM7	0.45	0.38–0.53	0.29	0.19–0.45
ITPKA	0.64	0.64–0.77	0.69	0.46–1.06
VCAN	0.30	0.30–0.36	0.25	0.16–0.39

Note: CI = confidence interval.

Wathlet. Cumulus gene expression and pregnancy. Fertil Steril 2012.

SUPPLEMENTAL TABLE 4

Mathematical models for prediction of pregnancy.

Biochemical pregnancy	
Only genes	$= -5.31 + 2.23 \times \text{EFNB2} - 0.759 \times \text{STC1} + 1.83 \times \text{CAMK1D}$
All factors	$= -3.58 + 2.56 \times \text{EFNB2} - 0.620 \times \text{STC1} + 2.15 \times \text{CAMK1D} - 0.0222 \times \text{relative } E_2$
Split-up dataset	$= -5.45 + 2.47 \times \text{EFNB2} - 0.593 \times \text{STC1} + 1.39 \times \text{CAMK1D}$
Live birth	
Only genes	$= -4.44 + 1.79 \times \text{EFNB2} - 0.458 \times \text{STC2} + 1.45 \times \text{CAMK1D}$
All factors	$= -6.28 + 1.73 \times \text{EFNB2} - 0.37 \times \text{STC2} + 1.30 \times \text{CAMK1D} + 0.180 \times$ days of stimulation

Wathlet. Cumulus gene expression and pregnancy. Fertil Steril 2012.