Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential

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Objective: To identify a gene expression signature in human cumulus cells (CCs) predictive of pregnancy outcome across multiple clinics, taking into account the clinic and patient variations inherent in IVF practice.
Design: Retrospective analysis of single human cumulus-oocyte complexes with the use of a combined microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) approach.
Setting: Multiple private IVF clinics.
Patient(s): Fifty-eight patients. Samples from 55 patients underwent qRT-PCR analysis, and samples from 27 patients resulted in live birth.
Intervention(s): Gene expression analysis for correlation with pregnancy outcome on individual human CCs collected immediately after oocyte retrieval. Pregnancy prediction analysis used leave-one-out cross-validation with weighted voting.
Main Outcome Measure(s): Combinatorial expression of 12 genes in 101 samples from 58 patients.
Result(s): We found a set of 12 genes predictive of pregnancy outcome based on their expression levels in CCs. This pregnancy prediction model had an accuracy of 78%, a sensitivity of 72%, a specificity of 84%, a positive predictive value of 81%, and a negative predictive value of 76%. Receiver operating characteristic analysis found an area under the curve of 0.763 ± 0.079, significantly greater than 0.5 (random chance prediction).
Conclusion(s): Gene expression analysis in human CCs should be considered in identifying oocytes with a high potential to lead to pregnancy in IVF-ET. (Fertil Steril® 2013;99:745–52. ©2013 by American Society for Reproductive Medicine.)
Key Words: Human cumulus cells, gene expression, pregnancy outcome, human oocyte, human embryo

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Received July 31, 2012; revised September 27, 2012; accepted October 13, 2012; published online November 29, 2012.
A.E.I. is an employee of and holds patents and stock in Gema Diagnostics. A.M.K. has had travel to meetings paid for by and holds patents in Gema Diagnostics. H.H.O. has received travel support and consulting and statistical review fees from Gema Diagnostics. P.R. has received travel support and consulting and statistical review fees from Gema Diagnostics. A.L. is a consultant for Gema Diagnostics. P.S. has nothing to disclose. M.S. has nothing to disclose. J.C.J. has nothing to disclose. J.C. has nothing to disclose. G.J.M.R. has nothing to disclose. E.F. has had travel to meetings paid for by, is a board member of, and owns patents and stock in Gema Diagnostics. R.G.R. has received travel support from, is a consultant for, and on the Scientific Advisor Board of Gema Diagnostics. J.B.C. has had travel to meetings paid for by, is a board member of, and owns patents and stock in Gema Diagnostics. J.A.C. has had travel to meetings paid for by, is a board member of, and owns patents and stock in Gema Diagnostics.
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Fertility and Sterility® Vol. 99, No. 3, March 1, 2013 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2012.10.041

The major hindrance to improving the efficiency of in vitro fertilization (IVF) has been the lack of an accurate objective method of selecting competent oocytes and embryos from among those capable of producing a healthy singleton pregnancy. Currently available selection tools rely almost exclusively on subjective and unreliable morphologic parameters. Although the transfer of multiple embryos can help to improve the chances of pregnancy, numerous significant health risks to mother and fetuses arise

during multiple gestation (1). Consequently, research has shifted toward universal single-embryo transfer (SET). A noninvasive tool that could objectively identify the most viable oocytes and embryos would improve pregnancy rates above current levels while decreasing the risk of multiple gestations.

The advent of the "omics" era has expanded our knowledge of the molecular processes surrounding human reproduction and holds great promise for treatments that target infertility. Specifically, much research has aimed to identify biomarkers indicative of embryo quality and pregnancy potential through proteomic, metabolomic, and transcriptomic approaches (2–6).

Of particular interest has been investigation into the transcriptional profile of cumulus cells (CCs), the specialized cells that surround and support the developing oocyte and are ordinarily discarded during the IVF process. CCs play a pivotal role in preparing the oocyte for ovulation, fertilization, and subsequent development via the bidirectional dialogue occurring between CCs and oocyte through intimately connected gap junctions; this dialogue involves signaling molecules, amino acids, essential metabolites (7, 8), and probably micro-RNAs (9).

Therefore, gene expression in CCs may provide an attractive method to noninvasively predict embryo quality and pregnancy potential. Indeed, several studies have shown a correlation between mRNA expression in CCs and oocyte and/or embryo quality (10–18), and differential expression of transcripts between CCs of oocytes that achieved pregnancy and those that did not (19–22). Hamel et al. reported that PGK1 and RGS2 expression predicted pregnancy in an intrapatient analysis, but the study fell short of extending the predictive ability of these genes to samples across a patient population (23).

Another important issue to take into account when designing gene expression analyses in samples where multiple factors may influence the outcome, is not only the sample size, but the inclusion of more than one site. Two recent studies focusing on the expression of 11 CC genes described SDC4, VCAN, EFNB2, and CAMK1D as predictors of pregnancy (24, 25). However, those studies included patients from only one clinical site, making it difficult to determine whether the signatures' prediction strength would hold true across a potentially more diverse patient population and whether non–intracytoplasmic sperm injection (ICSI) fertilization procedures would affect the prediction. It is also noteworthy that those studies used two stimulation protocols—a factor, as the same group reported, known to add variability to the expression of SDC4 and VCAN in CCs (18, 25). As the authors noted, interrogating a larger unrelated gene set may yield a pregnancy signature with more discriminating power.

The present study sought to identify gene expression biomarkers in human CCs that correlate with oocyte viability and the ability to produce a pregnancy. We performed gene expression analysis using a combination of microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) methods (Fig. 1). This study identified a novel set of 12 genes predictive of pregnancy outcome, which we generated from CCs from three clinical sites with the use of patient-specific stimulation protocols.

MATERIALS AND METHODS

Patient Selection, Implantation, and Pregnancy

This Institutional Review Board–approved retrospective study included patients undergoing either IVF or ICSI from one clinical site in Chile (Clinica Las Condes [CLC]) and two in the U.S. (Jarrett Fertility Group [JFG] and Pacific Fertility Center [PFC]). One, two, or three embryos were transferred to each patient, and embryo transfers occurred on day 2, 3, or 5. Clinical pregnancy, defined as the presence of fetal heartbeat and gestational sac by ultrasound examination, was determined 4–9 weeks after embryo transfer depending on the clinic's program. The Centers for Disease Control uses these as the standard criteria for defining pregnancy to report IVF results in the USA. The present study included only samples from patients for whom all embryos transferred resulted in pregnancy (P: full success) or patients for whom no embryos transferred resulted in pregnancy (N: no success). Live birth outcome was further recorded for patients with clinical pregnancy (P samples). We excluded patients older than 35 years, patients with fibroids >4 cm in diameter, those with a body mass index >35 kg/m^2, and those with a history of chemo- or radiotherapy. Additionally, this study excluded families with severe male factor infertility as defined by a total sperm count of <5 million or a history of testicular biopsy.

Patient Stimulation

Clinicians determined the most appropriate means for stimulating their patients, but protocols generally combined either GnRH agonist or GnRH antagonist, to suppress spontaneous ovulation, with purified or recombinant FSH; they also either did or did not include hMG or luteal phase support. Ovarian response and follicular development were monitored by serum E_2 level and transvaginal ultrasound. We induced final follicular maturation by administering hCG and retrieved oocytes with ultrasound guidance 36 hours later.

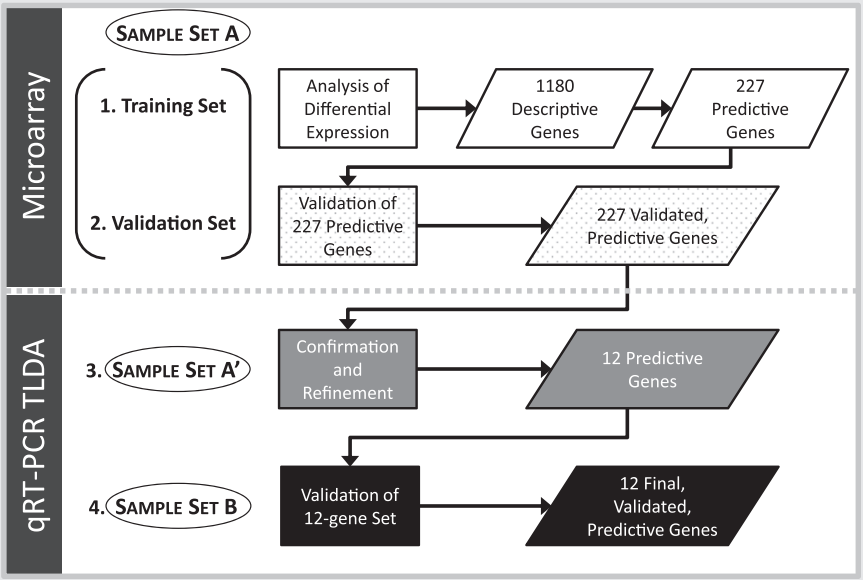
Human CC Collection

Individual cumulus-oocyte complexes (COCs) were rinsed in culture medium to remove any blood, loose cells, or other debris. A small number of CCs from each COC, were mechanically removed, careful to not take the very outer- or innermost layers. Each CC sample was rinsed in phosphate-buffered saline solution and placed in a microcentrifuge tube with 100 μL extraction buffer (Life Technologies) and resuspended gently by pipetting. Individual CC samples were incubated at 42°C for 30 minutes, centrifuged, and frozen in liquid nitrogen until they were shipped to a processing laboratory. Corresponding oocytes were placed in individual culture drops and cultured individually until embryo transfer (ET).

RNA Isolation

RNA isolation was performed with the use of the Picopure RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions. We analyzed total RNA quantity and quality with the use of a Nanodrop 2000 spectrophotometer (Nanodrop Technologies). Total RNA isolation was done at Michigan State University, East Lansing, and at Genemarkers in Kalamazoo, Michigan.

FIGURE 1



Study flowchart. Study design broken down by platform and cumulus cell (CC) sample sets. The first set of individual biologic samples (sample set A) was used on microarray to identify an initial candidate set of pregnancy predictive genes. A subset (sample set A′) of these microarray samples were then analyzed on quantitative reverse-transcription polymerase chain reaction Taqman Low-Density Array (qRT-PCR TLDA) to confirm and refine the panel of pregnancy predictive genes, resulting in a strongest and smallest set of 12 genes. This 12-gene signature was then validated on an entirely new biologic set of CCs (sample set B) from new patients.

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Microarray Analysis

We performed transcriptional profiling of 64 individual CC samples (29 P, 35 N; Table 1) from 36 patients with the use of Affymetrix HG-U 133 Plus 2.0 chips, which use >54,000 probe sets representing >47,000 transcripts and variants. We synthesized and amplified cDNA with the use of a protocol developed in house and previously described (26). Samples were analyzed withthe use of Affymetrix Genechip Microarray Analysis Suite 5.0 and Expression Console software (Affymetrix) for quality control assessment and normalization following the manufacturer's instructions.

Prediction Analysis

We applied a weighted voting approach utilizing "signal to noise ratio" (SNR) to assess predictor value of a gene g (27). This approach defines a neighborhood around ideal gene expression vectors for both P and N sample groups. SNR punishes genes with an expression highly deviant in either group and provides a signed ranking method for a gene's membership. In this case large positive SNR values indicate a good predictor for the P group and large negative values indicate a good predictor for the N group.

When we are given a predictor set of T genes, a group of P and N samples and a new sample S to be predicted, the vote of each gene represents how well the gene in sample S relates to the "behavior" of the gene in the P and N samples. If the gene vote is positive, we conclude that S is predicted to be P and if the gene vote is negative, we predict S as N. Cycling through all genes in the predictor set we obtain T votes used in the prediction of sample S.

When a prediction model is applied on a data set, the data set is first divided into training and validation sets. The predictor gene set is calculated on the training set with the use of leave-one-out cross-validation (L1OXV). In the L1OXV method using a predictive gene set of *T* genes, one sample in the training set is left-out and top *T* genes using the remaining samples that differentiate between N and P are calculated. Using these *T* genes, the sample that is left out is predicted as N or P. This process is cycled through all samples in the training set, leaving one out at a time. The total number of correct predictions is listed as the accuracy of the predictor on the training set. The predictor set of *T* genes is then applied in the validation set. We assigned significance of the predictor genes with the use of Fisher test and two additional strategies: 1) a permutation test, in which we randomly permuted class labels of P and N sample groups and identified optimum gene predictors using the same strategy; and 2) randomization test, in which we assessed the accuracy of *T* randomly chosen gene predictors with the use of the original data set class labels. We compared the performance of the original predictor set with the results obtained using permutation and randomization tests to assess the original predictor set's significance. In both tests, we used 1,000 realizations.

Quantitative Real-Time PCR

We performed cDNA synthesis using 8 ng total RNA and the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's protocol. Preamplification was done according to the Taqman Preamp Pools

TABLE 1

Patient and sample numbers by sample set and platform.

Samples (patients)							
Set A—microarray[a] n = 64 (36)							
Training		Validation		Set A′—qPCR[b] n = 49 (33)		Set B—qPCR[c] n = 37 (22)	
P	N	P	N	P	N	P	N
15 (14)	18 (16)	14 (12)	17 (15)	25 (16)	24 (17)	18 (11)	19 (11)

Note: N = nonpregnant samples; P = pregnant samples; qPCR = quantitative reverse-transcription polymerase chain reaction Taqman Low-Density Array.
[a] Set A: 64 samples first used on microarray to identify first set of 227 predictive genes. Most patients contributed sibling samples to both training and validation sets.
[b] Set A′: 49 samples (from the 64 used on microarray) used on qPCR TLDA to confirm and refine to 12 predictive genes.
[c] Set B: 37 new biologic samples used on qPCR TLDA to validate final 12-gene predictive set.

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Protocol (Life Technologies) using a custom Preamp Pool for 381 unique mRNA assays. Each sample reaction included 25 μL 2× Taqman Preamp Master Mix (Life Technologies), 12.5 μL custom Preamp Pool (Life Technologies), and 12.5 μL cDNA template. The thermocycler conditions were as follows: 10 minutes at 95°C, followed by 14 cycles of 15 seconds at 95°C and then 4 minutes at 60°C. We used a custom Taqman Low-Density Array (TLDA; Life Technologies) and ran one sample per array. Endogenous control genes 18S, GAPDH, and β-actin were included for relative quantification of transcripts. Forty-nine of the 64 individual CC samples previously used in the microarray, along with 37 new individual biologic CC samples from new patients, were analyzed on the TLDA (Table 1).

Statistics

We used the Genorm algorithm in Real-Time Statminer (Integromics) software to identify the most stable endogenous control gene or combination of endogenous control genes on the qRT-PCR TLDA across all sample sets. The Mann-Whitney test (28) was used to evaluate the clinical characteristics between P and N groups. Because we assessed several variables, we used $\alpha = .01$ to determine statistical significance so as to manage the potentially inflated false-positive error rate. Fisher exact test was used to determine the significance of prediction results during the pregnancy prediction analysis of the qRT-PCR gene expression data. We used analysis of variance (ANOVA) to assess categoric variable differences in gene expression, and we used Pearson correlation to evaluate the relationship between continuous variables and gene expression. Receiver operating characteristic (ROC) analysis was performed on the gene expression using the clinical pregnancy outcome (P, N) as the basis for truth. The ROC curve was created by plotting the true positive fraction (TPF, or sensitivity) versus the false positive fraction (FPF, or 1 − specificity) determined by moving the cutpoint value along the gene expression range. The area under this curve (AUC) indicates the degree of predictive ability of the gene expression, ranging from 0.5 (random chance) to 1.0 (perfect). All analyses were carried out with the use of SAS software (v. 9.2) or Medcalc (v. 11.3.1.0).

RESULTS

Patient and Sample Clinical Characteristics

The analysis included a total of 101 CC samples, 86 of which were included in the qRT-PCR TLDA, from 55 patients (Fig. 1, Table 1). All TLDA P samples that were confirmed as clinical pregnancies at fetal heartbeat check advanced to healthy live birth.

Of the 86 samples used to confirm, refine, and validate the predictive gene set with the use of qRT-PCR, 25, 45, and 16 samples were provided by CLC, JFG, and PFC, respectively (Supplemental Table 1, available online at www.fertstert.org). The majority of samples (n = 69) came from double ETs; eight CCs came from single ETs, and nine from triple ETs. ETs for 47 samples occurred on day 2 or 3, and for 39 on day 5; no significant difference existed between P and N groups regarding the day of ET. We found no differences in the primary clinical characteristics, such as oocyte age and cycle number, between P and N groups (Supplemental Table 2, available online at www.fertstert.org). However, we found a higher number of metaphase II (MII) oocytes ($P = .008$) in the P group and a lower fertilization rate (number of 2PN from MII oocytes; $P = .002$) in the P group (Supplemental Table 2). Owing to these observed differences between groups, we ran a clinical correlate of gene expression analysis, which we describe in a subsequent section.

Pregnancy Prediction Analysis

First, we used microarrays to obtain transcriptional profiling for 64 individual CC samples (35 N and 29 P; Table 1; Fig. 1). SNR was used to assess the predictive value of a gene with the use of weighted voting, as previously described (28). This group was divided into: 1) a training set (18 N and 15 P) to find a predictive set of genes; and 2) a validation set (17 N and 14 P). We used the validation set to test the performance of the predictive genes; the validation set was composed of samples that were not used in development of the predictive model. This strategy prevented overfitting and provided an assessment of the predictive signature's robustness (29). To find genes that correlated with success, we identified genes in the training set (P vs. N) that showed differential expression based on *t* tests ($P < .05$ with Bonferroni correction for multiple hypothesis testing). The resulting 1,180 genes, called "descriptive genes," were used for L1OXV in the training set (30). Weighted voting analysis revealed a 227-gene predictor set yielding 97% L1OXV accuracy (32/33 correct predictions; 17/18 N and 15/15 P correctly predicted) on the training set and 87% (27/31 correct predictions; 17/17 N and 10/14 P correctly predicted) prediction accuracy on the validation set. The prediction results remained significant with the use of Fisher test, permutation test, and randomization test ($P < .05$).

Validation and Refinement of Predictive Genes with qRT-PCR

Of 227 genes found to be predictive of pregnancy outcome, we included 196 in our custom TLDA for qRT-PCR validation. The endogenous controls β-actin, GAPDH, and 18S were evaluated for the most stable expression across the sample set. We found that 18S alone was most stable, and Ct values were normalized to that gene's expression level, providing dCt values which represented the fold change of a sample's gene relative to 18S expression.

We used a subset of 49 samples (24 N and 25 P; Table 1; Fig. 1) out of the 64 samples used in the microarray to confirm and further refine the predictive gene set. After normalization to 18S, we observed that 84 genes showed concordant expression on TLDA, as was previously determined on microarray with the same 49 biologic samples. The use of pregnancy prediction analysis on these 84 genes with the same strategy (weighted voting using SNR) yielded a predictive set of 12 genes. To further assess the predictive value of the 12-gene set, we ran the TLDA on 37 new biologic samples from patients (19 N and 18 P; Table 1; Fig. 1) not used in the microarray analysis. The predictor gene set remained significant with the use of the Fisher test, the permutation test, and the randomization test ($P<.05$) during both refinement and validation procedures.

Gene Expression in Cumulus Cells as a Biomarker of Pregnancy Outcome

The 12-gene predictor set identified with the use of qRT-PCR TLDA on sample set A′ (49 samples previously screened by microarray) was validated on sample set B (37 new biologic samples not used in microarray) with the use of weighted voting as previously described. Seven genes were up-regulated in P samples compared with N, and five genes were down-regulated in P compared with N (Supplemental Table 3, available online at www.fertstert.org). When applied to the validating B data set (37 samples), this pregnancy prediction model yielded an accuracy of 78%, a sensitivity for identifying successful pregnancy outcomes of 72%, a specificity for identifying failed pregnancy outcomes of 84%, a positive predictive value (PPV) of 81%, and a negative predictive value (NPV) of 76% (Table 2).

ROC analysis, a common method for evaluating the diagnostic utility of a test (31, 32), was conducted to determine the predictive power of identifying a successful pregnancy outcome based on the 12-gene prediction values for the validating 37 B samples (Table 3; Supplemental Fig. 1, available online at www.fertstert.org). The AUC, which indicates the degree of predictive ability, was 0.763 ± 0.079, which is significantly ($P=.0009$) greater than 0.5 (random chance prediction). Our sample size and the AUC observed in our ROC analysis fall in line with previous diagnostic reports within the IVF field (33, 34).

Clinical Correlates of Gene Expression

We evaluated patients' clinical characteristics for potential correlation with the 12-gene expression prediction values. Again, because several variables were being assessed, we used $\alpha = .01$ to determine statistical significance to manage the potentially inflated false-positive error rate. Of the continuous variables, none significantly correlated with the prediction value (Supplemental Table 4, available online at www.fertstert.org), including the number of MII oocytes and the fertilization rate (2PN/MII), despite their displaying different values between pregnant and nonpregnant samples. Although the number of MII oocytes and the fertilization rate differed significantly in the pregnancy outcome groups, neither variable correlated with the gene expression signature. That is, despite different numbers of MII oocytes and different fertilization rates between the P and N groups, this did not seem to affect the strength of the pregnancy signature.

The differences in the sum of the 12-gene prediction value for the categoric assessments were evaluated with the use of ANOVA. If the overall test for category differences was considered to be significant at $\alpha = .01$, we evaluated pairwise comparisons of the categories. Only two categoric variables, gonadotropin and ET catheter, were found to differ significantly in gene expression (Supplemental Table 5, available online at www.fertstert.org). Regarding gonadotropin, only JFG used the pFSH/hMG regimen (n = 45); PFC used rFSH exclusively (n = 16). Thus, we found a degree of confounding between site and gonadotropin, and these results should be interpreted with caution. Similarly, regarding the ET catheter, results should be interpreted cautiously, because a confounding effect resulted from each site using different catheters exclusively. Furthermore, the Wallace catheter sample size was very small (n = 5), providing very little power from which to draw conclusions. Finally, regarding clinical site, the majority of samples from CLC were collected much earlier and stored longer than those from JFG, likely explaining the difference seen in predictive values between these sites.

TABLE 2

Specific predictive accuracies of the 12-gene pregnancy signature on the validating sample set B.

Overall accuracy	78% (29/37)
Sensitivity	72% (13/18)
Specificity	84% (16/19)
Positive predictive value	81% (13/19)
Negative predictive value	76% (16/18)
Odds ratio for successful outcome (95% CI)	13.9 (2.8–69.2)
P value (odds ratio = 1)	.0006

Note: Percentages refer to number of fetal heartbeats per number of embryos transferred. Eighty-six cumulus samples were screened. The overall accuracy was defined as true predictions/(true + false predictions). Sensitivity was defined as true positives/(true positives + false negatives), and specificity was defined as true negatives/(true negatives + false positives). Positive predictive value was defined as the proportion of embryos predicted as successful that implanted, and negative predictive value was defined as the proportion of embryos predicted as unsuccessful that failed to implant.

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DISCUSSION

The ability to select viable oocytes and embryos during IVF has significant medical, social, and financial benefits. A diagnostic assay using CCs to complement morphology would present a noninvasive approach to attaining this goal. A critical question, however, has remained whether developing a test robust enough to overcome inherent variations in patients and clinics would be possible. The present report

TABLE 3

Predictive power of the 12-gene prediction values.

	Combined sample sets A′ + B	Sample set A′	Validating sample set B
Successes/failures	43/43	25/24	18/19
AUC ± SE	0.725 ± 0.055	0.703 ± 0.075	0.763 ± 0.079
95% CI	0.618–0.816	0.556–0.825	0.595–0.887
Prob. (AUC = 0.5)[a]	<.0001	.0067	.0009
Sensitivity at threshold	65%	56%	72%
Specificity at threshold	77%	79%	84%

Note: Percentages refer to number of fetal heartbeats per number of embryos transferred. AUC = area under the receiver operating characteristic curve; CI = confidence interval.
[a] Degree of predictive ability (*P* value) significantly greater than 0.5 (random chance prediction).

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describes, for the first time, a novel set of 12 genes—produced from multiple sites and diverse clinical protocols—that predict pregnancy outcome. Our proposed prediction strategy, based on the expression levels of the genes in CCs, paves the way for a noninvasive supplementary tool for selecting viable oocytes. We developed the predictive gene set with the use of a global expression profiling approach and then used qRT-PCR to validate it on two independent biologic sample sets. Additional ROC analysis confirmed that this predictive gene set had significant predictive power.

Although the genes that ultimately comprised our final gene set do not overlap with genes previously reported as predictive of pregnancy, this is not entirely surprising. This could be due to several factors: differences in technical approaches, such as the use of TLDAs, the fact that our algorithm incorporates weighted voting, which places varied contribution of each gene's expression in the prediction model, or a combination of both.

The genes in our predictive set are, in part, involved with glucose metabolism, transcriptional regulation, gonadotropin regulation, and apoptosis—all essential to viable COC processes. Considering the generally known functions of some of the genes or gene families, it is not improbable that they could reveal themselves as part of a pregnancy-predictive CC gene panel. For example, the fibroblast growth factor (FGF) family plays an important role in regulating cell survival, and FGF-12 was up-regulated in our P group compared with the N group of samples.

Glucose, which is metabolized by the glycolysis pathway, acts as a crucial metabolite for the COC (4). The breakdown of glucose by CCs provides the oocyte with essential nutrients, such as pyruvate and lactate, to complete maturation in preparation for ovulation. Converting glucose into these byproducts has further importance: providing the oocyte with the maternal store of metabolites/energy sources as it is nurtured by the surrounding granulosa cells, of which CCs are one type. Thus, granulosa cells play a critical role in supporting the developing oocyte and establishing its maternal supply of energy resources to carry it through the first few cell divisions (35). SCL2A9 (also known as GLUT9), a member of the SLC2A facilitative transporter family, plays an important role in glucose homeostasis (36). Specifically, SCL2A9 has been demonstrated to transport uric acid and hexose sugars, of which glucose is one example (37). In a bovine model, mature COCs were observed to use more glucose and its metabolic products than were immature COCs (38). Given this fact, the increased expression of SCL2A9 in CCs corresponding to viable oocytes may reflect a more dynamic transport of glucose within those CCs and therefore a more properly functioning metabolic state in the COCs as a whole.

NR2F6 also was up-regulated in our P sample sets compared with N. This gene is an orphan nuclear receptor, belonging to a subgroup of the nuclear receptor superfamily of transcription factors and cofactors. Although the exact function of NR2F6 remains undefined in CCs, orphan nuclear receptors are known to play a role in many reproductive processes (39). Specifically, research has shown that NR2F6 inhibits LH receptor (LHr) transcription via promoter repression (40). The formation of LHr on the surface of CCs plays a key part in proper follicular maturation before the LH surge, which induces ovulation. However, overexpression of LHr can have adverse effects on the ovulatory process; higher levels of this receptor have been reported in the granulosa cells of women with polycystic ovaries compared with those without (41). The slightly lower expression of NR2F6 seen in our N group may indicate a hyperactive state of LHr expression, which could lead to suboptimal maturation of the follicle.

We found four additional genes that were up-regulated in the CCs of P compared with N samples: ARID1B, FAM36A, GPR137B, and ZNF132. ARID1B is part of the SWI/SNF chromatin remodeling complex, which plays a critical role in cell cycle control. Research has demonstrated the necessity of open gap junction communication between follicular cells and their oocyte for proper meiotic maturation, which involves chromatin remodeling maturation (42). Increased ARID1B in our P samples may facilitate gap junction communication and improve oocyte viability. The function of FAM36A is not well characterized, but this protein has been localized in mitochondria and is integral to the membrane. GPR137B is also poorly characterized; however, this gene encodes a G-protein–coupled receptor (GPCR) integral membrane protein. Given the prominent role that GPCRs play in interpreting external messages for a cell, this could indicate an important role for GPR137B in signaling within the follicular microenvironment. ZNF132—yet another gene with a poorly understood function—is a member of the zinc finger protein family, which aids in directly affecting transcription by acting as the DNA-binding subunit of transcription factors, thus conferring DNA sequence specificity.

Five genes in our signature were down-regulated in P compared with N samples: DNAJC15, RHBDL2, MTUS1, NUP133, and ZNF93. Little is known about the specific actions of these genes. DNAJC15 is localized to mitochondria and membranes and is thought to have heat shock–binding properties. RHBDL2 is an intermembrane protease, and research increasingly suggests the importance of intermembrane proteolysis in regulating a variety of cellular processes, such as development and metabolism (43). MTUS1 has previously been reported as more highly expressed in ovaries than in other tissues (44), although the specific action of this gene in ovarian regions remains undefined. NUP133 is involved with nucleocytoplasmic transport activity, a subset of which includes glucose transport. Finally, ZNF93, another zinc finger gene, has an as-yet-undescribed function but is thought, like other characterized zinc finger proteins, to regulate transcription in a direct manner as the DNA-binding component of transcription factors.

The functional roles of each gene in our predictive set regarding oocyte and embryo viability remain to be elucidated. Hypothesis-driven experiments are required to investigate how each gene expressed in CCs acts individually, and in combination, to impart or compromise the developmental competence of their respective oocyte, dependent on its level of expression.

Despite a significant difference in the number of MII oocytes and the fertilization rate between samples from pregnant and nonpregnant patients, the clinical correlates of gene expression analysis has demonstrated that these differences have no correlation with the gene expression values, and therefore no effect on the strength of our predictive gene set.

The effect on gene expression values identified in gonadotropin choice and ET catheter between pregnancy outcome groups appears to be more indicative of the clinical site, because usage of these factors were confounded with site. Again, regarding the clinical site difference seen between CLC and JFG, the majority of samples from CLC were collected earlier and stored longer than those from the JFG, which likely explains the difference seen in this covariate.

CONCLUSION

The data presented herein reveal a novel 12-gene set in CCs that are predictive of pregnancy; these data, from multiple sites using multiple stimulation protocols, had an overall accuracy of 78%. ROC analysis confirmed the predictive power of our test, with an AUC of 0.763 ± 0.079, which is significantly ($P=.0009$) greater than the 0.5 of random chance prediction and compatible with the expectations for a successful diagnostic test. This is particularly promising given the heterogeneous nature of the patients and the different treatments they received.

The next step will be to apply this gene signature prospectively to a randomized control clinical trial across multiple sites to confirm its pregnancy prediction value in identifying the oocytes with the highest pregnancy potential for embryo transfer.

Acknowledgments: The authors thank Elpida Fragouli, Doug Powers, and Dagan Wells for their thoughtful review of the manuscript. The technical assistance of Libby Lehigh and Neli Ragina is also gratefully appreciated.

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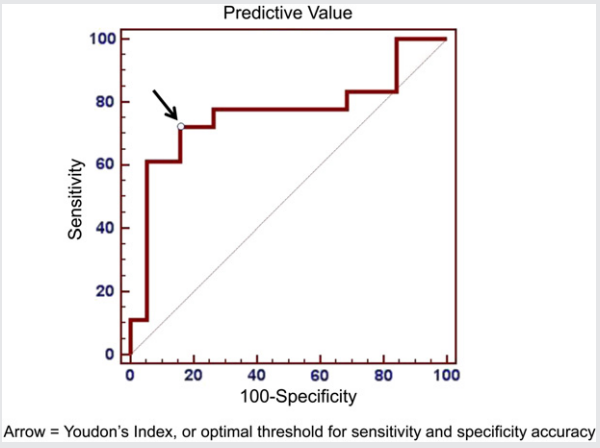
SUPPLEMENTAL FIGURE 1



Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic utility of the predictive power of identifying a successful pregnancy outcome based on the 12-gene prediction values for the validating sample set B. The *open circle* (*arrow*) on the curve is the Youdon index, or optimal threshold, which minimizes misclassification of prediction.

lager. Cumulus gene expression and pregnancy. Fertil Steril 2013.

SUPPLEMENTAL TABLE 1

qRT-PCR patient and sample numbers by clinic.

	Samples (patients); n = 86 (55)		
	P	N	Total
CLC	8 (14)	11 (8)	25 (16)
JFG	20 (12)	25 (15)	45 (27)
PFC	9 (7)	7 (5)	16 (12)
Total	43 (27)	43 (28)	86 (55)

Note: The qRT-PCR analysis included a total of 86 cumulus samples from 55 patients. Three sites participated in this study: CLC (Clinica Las Condes), JFG (Jarrett Fertility Group), and PFC (Pacific Fertility Center). N = nonpregnant samples; P = pregnant samples; qRT-PCR = quantitative reverse-transcription polymerase chain reaction.

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SUPPLEMENTAL TABLE 2

qRT-PCR sample clinical characteristics.

		P (n = 43)		N (n = 43)		
Variable	**Unit**	**Mean**	**SD**	**Mean**	**SD**	***P* value**
Oocyte Age	y	31.26	0.50	29.53	0.63	.675
BMI	kg/m^2	23.27	0.58	23.38	0.56	.572
IVF cycle	n	1.44	0.13	1.37	0.07	.573
Oocytes retrieved (OR)	n	12.74	1.15	10.44	0.95	.156
MII oocytes	n	10.16	0.94	7.23	0.76	.008[a]
Oocyte maturity	%	82.46	3.67	74.37	4.19	.149
2PN	n	7.40	0.66	5.72	0.59	.056
Fertilization rate[b] (2PN/ER)	%	61.86	3.46	60.76	4.03	.856
Fertilization rate[b] (2PN/MII)	%	74.54	2.30	83.92	3.11	.002[a]
Day of ET	n	3.91	0.18	3.63	0.18	.276

Note: 2PN = two pronuclei; BMI = body mass index; ET = embryo transfer; MII = metaphase II; other abbreviations as in Supplemental Table 1.
[a] Significant difference between P and N groups.
[b] Statistics were run after first calculating the rates for each patient individually.

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SUPPLEMENTAL TABLE 3

Set of 12 genes used to predict pregnancy outcome.

Gene symbol	Gene name	P vs. N (fold change)	Known or suggested function[a]
FGF12	Fibroblast growth factor 12	Up (1.52)	The FGF family is involved in an array of biologic processes including cell growth, morphogenesis, embryonic development, and tissue repair
GPR137B	G-protein–coupled receptor 13b	Up (1.31)	G-protein–coupled receptor (GPCR) family is integral membrane proteins and play a prominent role in interpreting external messages for a cell and inducing signaling cascades within the cell
SLC2A9	Solute carrier family 2 (facilitated glucose transporter), member 9	Up (1.26)	The SLC2A family plays significant roles in maintaining glucose homeostasis. This gene facilitates glucose transport.
ARID1B	AT-rich interactive domain 1B (SWI1-like)	Up (1.57)	Chromatin remodeling–dependent transcriptional regulation
NR2F6	Nuclear receptor subfamily 2, group F, member 6	Up (1.15)	Inhibits human LH receptor transcription
ZNF132	Zinc finger protein 132	Up (1.08)	Zinc finger proteins assist in directly affecting transcription by conferring DNA sequence specificity as the DNA-binding domain of multisubunit transcription factors
FAM36A	Family with sequence similarity 36, member A	Up (1.32)	Unknown function but integral membrane and mitochondrial localization
ZNF93	Zinc finger protein 93	Down (−1.62)	Zinc finger proteins assist in directly affecting transcription by conferring DNA sequence specificity as the DNA-binding domain of multisubunit transcription factors
RHBDL2	Rhomboid, veinlike 2 (Drosophila)	Down (−1.11)	An intermembrane protease; intermembrane proteolysis is progressively being more recognized as participating in regulation of a host of cellular processes, such as development and metabolism
DNAJC15	DnaJ (Hsp40) homologue, subfamily C, member 15	Down (−6.52)	Localized to mitochondria and membrane, and thought to have heat shock–binding properties
MTUS1	Microtubule-associated tumor suppressor 1	Down (−1.42)	Identified as highly expressed in ovary compared with other tissues, but its function in this region in unknown
NUP133	Nucleoporin 133kDa	Down (−1.28)	Nucleocytoplasmic transport activity

Note: The final set of 12 genes that rendered the strongest smallest set predictive of pregnancy was derived through qRT-PCR TLDA analysis. Abbreviations as in Supplemental Table 1.

[a] http://www.ncbi.nlm.nih.gov/gene/.

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SUPPLEMENTAL TABLE 4

Continuous variable correlation with prediction value.

	Correlation	*P* value (corr. = 0)
Oocyte age	−0.14	.1986
BMI	−0.09	.4532
No. of follicles	0.06	.5640
No. of oocytes retrieved (OR)	−0.07	.5444
No. of mature oocytes (MII)	−0.15	.1600
No. of oocytes fertilized (2PN)	−0.14	.2016
Fertilization rate (2PN/OR)	−0.10	.3361
Fertilization rate (2PN/MII)	0.07	.5228

Note: Abbreviations as in Supplemental Table 2.

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SUPPLEMENTAL TABLE 5

Categoric variable correlation with prediction value.

	P value for overall differences	Significant pairwise comparisons (n)
Site	.0133	CLC (25) vs. JFG (45): *P*=.0034
GnRH analogue	.0970	
Gonadotropin	.0030[a]	pFSH/hMG (28) vs. rFSH (19): *P*=.0081 pFSH/hMG (28) vs. rFSH/hMG (39): *P*=.0014
Fertilization	.3605	
ET catheter	.0016[a]	Wallace (5) vs. Frydman (13): *P*=.0010 Wallace (5) vs. Cook (11): *P*=.0152 Wallace (5) vs. soft-echo (12): *P*=.0426 USP (46) vs. Frydman (13): *P*=.0006
Luteal phase support	.4261	
ET day	.0235	
IVF cycle	.1367	
No. of embryos transferred	.0361	

Note: We used ANOVA to evaluate overall differences in the sum of 12-gene prediction value for the categoric assessments. When the overall test for category was significantly different ($\alpha = 0.01$), we then evaluated pairwise comparisons of the categories. ET = embryo transfer.

[a] Significant difference between P and N groups.

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