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ASSISTED REPRODUCTION TECHNOLOGIES

# Protein expression in human cumulus cells as an indicator of blastocyst formation and pregnancy success

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**Abstract**
*Purpose* The goal for the present study was to implement a technique for protein extraction and identification in human cumulus cells (CCs).
*Methods* Forty samples of CCs were collected after ovum pick-up from patients undergoing intracytoplasmic sperm injection (ICSI). Samples were split into the blastocyst group ($n = 10$), including patients in which all embryos converted into blastocysts, and the non-blastocyst group ($n = 10$), including patients in which none of the embryos reached the blastocyst stage or the positive-pregnancy ($n = 10$) and negative-pregnancy group ($n = 10$). Proteins were extracted and injected into a liquid chromatography system coupled to a mass spectrometer. The spectra were processed and used to search a database.
*Results* There were 87 different proteins in samples from the blastocyst and non-blastocyst groups, in which 30 were exclusively expressed in the blastocyst group and 17 in the non-blastocyst group. Among the 72 proteins detected in the pregnancy groups, 19 were exclusively expressed in the positive, and 16 were exclusively expressed in the negative-pregnancy group.
*Conclusions* CC proteomics may be useful for predicting pregnancy success and the identification of patients that should be included in extended embryo culture programs.

**Keywords** Cumulus cell · Blastocyst · Mass spectrometry · Pregnancy · Proteomics

*Capsule* CC proteomics may be useful for predicting pregnancy success and the identification of patients that should be included in extended embryo culture programs.

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## Introduction

Since the first successful in vitro fertilization (IVF) [1], assisted reproduction techniques (ART) have advanced considerably. Despite these advances, it has been reported that around 70–80 % of embryos produced in vitro fail to implant, and 66 % of IVF cycles fail to result in pregnancy [2]. The low efficiency contributes to the practice of multiple embryo transfer, which seeks to overcome implantation failure and maximize pregnancy rates. Unfortunately, this procedure frequently leads to the inconvenient outcome of multiple pregnancies [3, 4].

A key step in ART is the assessment of gamete and embryo viability to identify the embryo(s) that are most likely to result in a pregnancy. Nevertheless, our knowledge of the molecular determinants of embryo viability is limited, and therefore, current embryo assessment strategies are based on embryo morphology.

Prolonging the duration of culture to day 5 and transfer of blastocyst stage embryos is associated with increased

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implantation rates compared with the transfer of cleavage stage embryos [5, 6]. Embryo transfer on day 5 allows chromosomally competent embryos to develop to the blastocyst stage and enables a better selection of embryos that have the potential for continued development, because the laboratory assessment occurs after the expression of the embryonic genome [7]. Moreover, transfer of embryos on day 5 has the advantage of physiological synchronization between endometrium function and embryonic development [8, 9]. Single blastocyst transfers have the additional advantages of increasing pregnancy rates and reducing multiple gestations because of their high implantation rates [10, 11].

While blastocyst transfer is beneficial in good prognosis patients, the same is not true for unselected groups of patients [12]. Therefore, some assisted reproduction centers may avoid adopting extended embryo cultures due to an unpredictable rate of blastocyst development.

Therefore, the development of reliable and non-invasive methods is of pivotal importance when selecting patients who could benefit from extended embryo culture programs. In the post-genomic era, many "omics" efforts are focusing on increasing our understanding of the relationships between the genome, DNA transcripts, proteins, metabolites, and phenotypes in cells and organisms [13]. Non-invasive approaches for embryonic development potential have the advantages of increasing the knowledge of embryo physiology, therefore allowing the development of methods to predict developmental competence and viability [14]. These approaches include genomic and proteomic profiling and analytical examination of the embryonic metabolome [13, 15–20].

Bidirectional communication between the oocyte and surrounding cumulus cells (CCs) is essential for the acquisition of oocyte competence [21]. It has been previously reported that because of their close connection with the oocyte, CCs may retain a footprint of the follicular conditions experienced by the oocyte [22].

The knowledge that CCs have a central role in the support of oocyte development and maturation has led various groups to focus their research on the analysis of CC gene expression (for review, see [23]). Although many studies have focused on the analysis of CC gene expression in the search for novel markers of oocyte competence and pregnancy outcome [14, 24–28], this approach does not actually reflect the cell phenotype. In fact, protein synthesis is the major outcome of gene expression and is directly associated with the observed phenotype, which is not always the case with RNA. Therefore, it is clear that protein analysis should be the preferred end-point of all physiological analyses.

Earlier embryonic proteomic studies utilized two-dimensional (2D) gel electrophoresis in combination with analysis of gel images [29, 30]. For known proteins [31] or to correlate protein phosphorylation with embryonic development [32], Western blot analysis has been used. More recently, mass spectrometry (MS) fingerprinting has been demonstrated to provide a reliable approach for the identification of groups of proteins within limited amounts of samples [33, 34].

The goal for the present study was to utilize the analytical power of MS with minimal sample preparation and minute analysis to identify patients that would benefit from extended embryo culture programs and to predict the pregnancy outcome by differential protein expression in CCs.

## Materials and methods

### Samples

CC samples were collected and stored at −20 °C, immediately after ovum pick-up from 40 patients undergoing intracytoplasmic sperm injection (ICSI) with embryo transfer performed on day 5. Samples were split according to blastocyst formation rate and pregnancy outcome.

There were two experimental groups based on blastocyst formation rate. The blastocyst group ($n = 10$) included patients in which all embryos had formed blastocysts. The non-blastocyst group ($n = 10$) included patients in which none of the embryos had reached the blastocyst stage. There were also two experimental groups considering the pregnancy outcome, including the positive-pregnancy group ($n = 10$) and negative-pregnancy group ($n = 10$).

The inclusion criteria were as follows: (i) ICSI cycle using ejaculated sperm with embryo transfer performed on day 5; (ii) patient's age (≤35 years old); (iii) number of retrieved mature oocytes (MII) ≥ 4; (iv) patients without pelvic and/or uterine abnormalities of clinical significance, absence of endometriosis grade III and IV, absence of polycystic ovary syndrome (PCOS); and (v) the presence of both ovaries. All of the cases with severe spermatogenic alterations were excluded from the study.

To meet the inclusion criteria and reach the number of samples in each group, 182 CC samples were collected.

Written informed consent in which patients agreed to share the outcomes of their cycles for research purposes was obtained. The study was conducted in accordance with the principles set out in the Declaration of Helsinki and was approved by the local institutional review board.

### Controlled ovarian stimulation and oocyte retrieval

Controlled ovarian stimulation was achieved by using recombinant FSH (Gonal-F; Serono, Geneva, Switzerland), as a daily dose, beginning on day 3 of the cycle. Pituitary blockage was performed by using a GnRH antagonist (Cetrotide, Serono, Geneva, Switzerland), when at least one follicle of at least 14 mm was visualized.

Follicular growth was monitored using transvaginal ultrasound examination starting on day 4 of gonadotropin administration. When adequate follicular growth and serum 17β estradiol levels were observed, recombinant hCG (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger the final follicular maturation. The oocytes were collected 35 h after hCG administration through transvaginal ultrasound ovum pick-up.

### Sample collection, preparation of oocytes, and morphological assessment

Immediately after follicle aspiration, the CCs were collected by using two 18-G needles coupled to 5-mL syringes under a stereomicroscope. The samples were stored at −20 °C, and oocytes were maintained in culture medium (Global® for Fertilization, LifeGlobal, Connecticut, USA) supplemented with 10 % human synthetic albumin (HSA, Irvine Scientific, Santa Ana, USA), which was covered with mineral oil (Ovoil™, Vitrolife, Kungsbacka, Sweden) and stored at 37 °C and 6 % $CO_2$ for 5 h.

The surrounding CCs that could not be manually removed were removed by exposure to a HEPES-buffered medium containing hyaluronidase (80 IU/mL, Irvine Scientific, Santa Ana, USA). The remaining CCs were then mechanically removed by gentle pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA). Oocyte morphology was assessed immediately prior to sperm injection using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon®, Tokyo, Japan) with a Hoffmann modulation contrast system under ×400 magnification. Oocytes that had released their first polar body were considered to be matured and were used for ICSI.

### Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed on all MII oocytes using the technique described by Palermo et al. [35]. The oocytes were individually placed in 4-μL droplets of buffered medium (Global® w/HEPES, LifeGlobal, Connecticut, USA), and the sperm was placed in a central 4-μL droplet of polyvinylpyrrolidone solution (PVP, Irvine Scientific, Santa Ana, USA) in a 50 × 40-mm glass culture dish (WillCo-dish®, New Jersey, USA) covered with warm mineral oil (Ovoil™, Vitrolife, Kungsbacka, Sweden), on a heated stage (37.0 ± 0.5 °C) of an inverted microscope.

### Assessment of fertilization, embryo quality, and embryo transfer

After the ICSI procedure, the presumptive embryos were individually maintained in a 50-μL drop of culture medium (Global®, LifeGlobal, Connecticut, USA) supplemented with 10 % human serum albumin (HAS) and covered with mineral oil in a humidified atmosphere with 6 % $CO_2$ at 37 °C until transfer, which occurred on the fifth day of development.

Approximately 18 h after ICSI, fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Subsequently, embryos were transferred to new drops of culture medium to be individually cultured for 48 h. The quality of the embryos was evaluated under an inverted microscope. High-quality embryos were defined as having the following characteristics: 8–10 cells on the third day of development, less than 15 % fragmentation, symmetric blastomeres, and the absence of multinucleation and zona pellucida dysmorphisms. Embryos lacking any of the above characteristics were considered to be of medium or low quality.

Up to two embryos from each couple were transferred to the patient. In special cases of low morphological quality, three embryos were transferred. Embryo selection for transfer was based on embryo and oocyte morphology.

### Clinical follow-up

Ten days after embryo transfer, the quantitative measurement of serum levels of the beta subunit of human chorionic gonadotropin (β-hCG), which is indicative of positive pregnancy, was performed. A clinical pregnancy was defined by the detection of a gestational sac and fetal heartbeat by pelvic transvaginal ultrasonography, performed between 3 and 4 weeks after embryo transfer.

### Sample preparation for mass spectrometry

To obtain the minimum amount of material for the study, CC samples from each group were pooled. Each pool was centrifuged at 3000×*g* for 15 min in order to discard the supernatant and concentrate the cells on the microtube wall, by specific positioning of microtubes in the centrifuge. Then, the cells were subjected to lysis by adding 100 μL of sample loading buffer (0.5 M Tris-HCL [pH 6.8], 10 % SDS, Glycerol, Bromophenol blue, DTT, Water—GE Healthcare, Piscataway, NJ) to each sample, followed by manual pipetting to obtain the lysates. The resulting lysates in the sample loading buffer were homogenized for 1 min and heated at 100 °C for 5 min for subsequent electrophoresis for protein concentration in the gels.

The proteins were concentrated in the gels in duplicate using 10 % (*v*/*v*) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and protein detection in gel was performed using Coomassie Brilliant Blue staining.

For protein band excision from the gels, the bands with a minimum size of 2 mm × 5 mm and intensively stained by Coomassie Brilliant Blue were cut into cubes (1 × 1 mm) and transferred to microtubes, according to the respective samples. For each sample, one of the

replicates had the albumin removed by band excision, guided by Amersham ECL full-range rainbow molecular weight marker (GE Healthcare, Piscataway, NJ). Gel piece destaining, reduction and alkylation, digestion of band pieces, and excision of peptides were performed as described by the Lamond Laboratory protocol (see http://www.lamondlab.com/pdf/LLingeldigestion.pdf).

The peptide quantification was carried out using the Bicinchoninic acid assay, after protein digestion and MS. For this purpose, 2 μL of sample containing the peptides was used in order to evaluate the efficiency of the protein extraction method.

During all the process of sample preparation, the following cautions were taken in order to prevent keratin contamination: (i) the glassware was washed with Extran and milliQ water followed by rinse with methanol; (ii) all the solutions were prepared right before using; (iii) the staining tray was washed and covered during gel staining; (iv) personnel working on the sample preparation were properly dresses with lab coat and gloves.

### Liquid chromatography and mass spectrometry

LC-MS/MS analyses were performed with digested protein samples (15 μL) in an Agilent 1290 Infinity LC System coupled to 6550 iFunnel Q-TOF LC/MS. The chromatographic conditions were as follows: POROSHELL 120 EC, C-18 column (Agilent 100 mm × 2.1 mm × 2.7 μM) maintained at 60 °C, with a flow rate of 0.5 μL/min with water, 0.1 % formic acid in water (A), and 0.1 % formic acid in acetonitrile (B). The elution gradients were as follows: 0 to 20 min—10 to 35 % B; 20 to 28 min—35 to 90 % B; and 28 to 30 min—90 % B in isocratic mode, resulting in 30 min of time acquisition. After chromatographic separation, the samples were subjected to MS in the Agilent Dual JetStream ionization source, in positive ion mode at 35 psi of nebulizer gas and 3.5 kV of capillary voltage. Full scan mass spectra were acquired with a scan range of 300–1700 *m/z* (8 spectra) and for MS/MS spectra with a scan range of 50–1700 *m/z* (4 spectra). The data obtained from LC/MS were analyzed using the Agilent MassHunter Qualitative Analysis Software B.06.

### Data analysis

The Agilent Mass Hunter Qualitative raw data were saved in .mgf format and processed using Mascot (Matrix Science, London, UK). Spectra were processed using Mascot Distiller, under conditions optimized for the Agilent Mass Spectrometer, and processed results were used in database searches using a reviewed SwissProt Human database, considering the use of trypsin, and a maximum of two missed cleavages. Carbamidomethylation was set as a fixed modification, and methionine oxidation as a variable modification. A maximum mass error of 30 ppm was allowed for MS and of 1 Da for MS/MS. Proteins were considered identified if they presented a maximum $p$ value of less than 5 %. A reverse database was used for estimation of false-positive rates. Finally, quantification was achieved using Exponentially Modified Protein Abundance Index (emPAI), which is a function based on the number of observed and observable peptides for any given protein [36].

## Results

### Blastocyst formation chance

The comparison of patients and cycles' characteristics between the blastocyst and non-blastocyst groups is described in Table 1.

Analysis of samples from the blastocyst and non-blastocyst groups revealed 87 different proteins. Most of the detected proteins were binding proteins, followed by enzymes. However, structural, transport, construction, and DNA repair proteins were also identified (Fig. 1).

Among the 87 detected proteins, 30 were exclusively expressed in the blastocyst group, and 17 were exclusively expressed in the non-blastocyst group. Forty proteins were expressed in both groups, and six of these were equally expressed and 34 were differentially expressed (Tables 2 and 3). From the proteins differentially expressed between the blastocyst and non-blastocyst groups, 23 proteins were highly expressed in the blastocyst group, and 11 proteins were highly expressed in the non-blastocyst group (Fig. 2).

### Pregnancy outcome

The comparison of patients and cycles' characteristics between the positive-pregnancy and negative-pregnancy groups are described in Table 4.

Analysis of samples from the positive-pregnancy and negative-pregnancy groups revealed 72 proteins. Similar to the embryo quality groups, most of the detected proteins were binding proteins, followed by enzymes. Construction, growth factors, anticoagulant, and DNA repair proteins were also detected (Fig. 3).

Among the 72 detected proteins, 19 were exclusively expressed in the positive-pregnancy group, and 16 were exclusively expressed in the negative-pregnancy group. Thirty-eight proteins were expressed in both groups, and 16 of these were equally expressed and 22 were differentially expressed (Tables 5 and 6). Among the proteins expressed among the positive- and negative-pregnancy groups, 16 proteins were highly expressed in the positive-pregnancy group and six proteins were highly expressed in the negative-pregnancy group (Fig. 4).

**Table 1** Comparison of patients and cycles' characteristics between the blastocyst and non-blastocyst groups

| Variables | Blastocyst group | Non-blastocyst group | *p* value |
|---|---|---|---|
| Maternal age (year) | 34.1 ± 1.8 | 33.3 ± 4.3 | 0.485 |
| Paternal age (year) | 38.1 ± 3.3 | 36.5 ± 3.8 | 0.325 |
| FSH administered (IU) | 2244 ± 612 | 2128 ± 425 | 0.771 |
| Estradiol level (pg/mL) | 1654 ± 1675 | 1354 ± 1354 | 0.548 |
| Number of aspirated follicles | 18.4 ± 8.1 | 20.3 ± 10.0 | 0.451 |
| Number of retrieved oocytes | 14.3 ± 7.5 | 13.8 ± 4.0 | 0.683 |
| Number of MII oocytes | 11.8 ± 4.9 | 9.7 ± 5.4 | 0.532 |
| Fertilisation rate % | 83.2 ± 15.1 | 78.3 ± 18.4 | 0.913 |

Note: values are mean + SD, unless otherwise noted. NS

## Discussion

In the present study, a number of proteins were detected in the human CCs by LC-MS. Some proteins were differently expressed in the CCs of patients in which all the embryos achieved the blastocyst stage when compared with those in which no embryo achieved this stage, and some proteins were differently expressed in the CCs of patients that achieved pregnancy when compared with patients that did not achieve the same outcome.

CCs are in direct contact with the oocyte, and an intense bi-directional communication occurs between them during folliculogenesis, which is essential for oocyte development. Therefore, the majority of investigations have focused on the analysis of CC gene expression to identify non-invasive predictors of embryo development and treatment outcome [14, 24–28]. However, few experiments have focused on protein expression [37], which is a more reliable indicator of the cellular phenotype.

In a previous experiment, Hamamah et al. [37] compared the protein expression profile of human CCs in relation to oocyte fertilization and ovarian stimulation protocol by 2D polyacrylamide gel electrophoresis. The study showed that less than 20 proteins differed between CCs surrounding fertilized oocytes and those surrounding non-fertilized oocytes; however, the proteins were not identified in this procedure.

A significant implication of the present study is the identification of potential biomarker candidates for

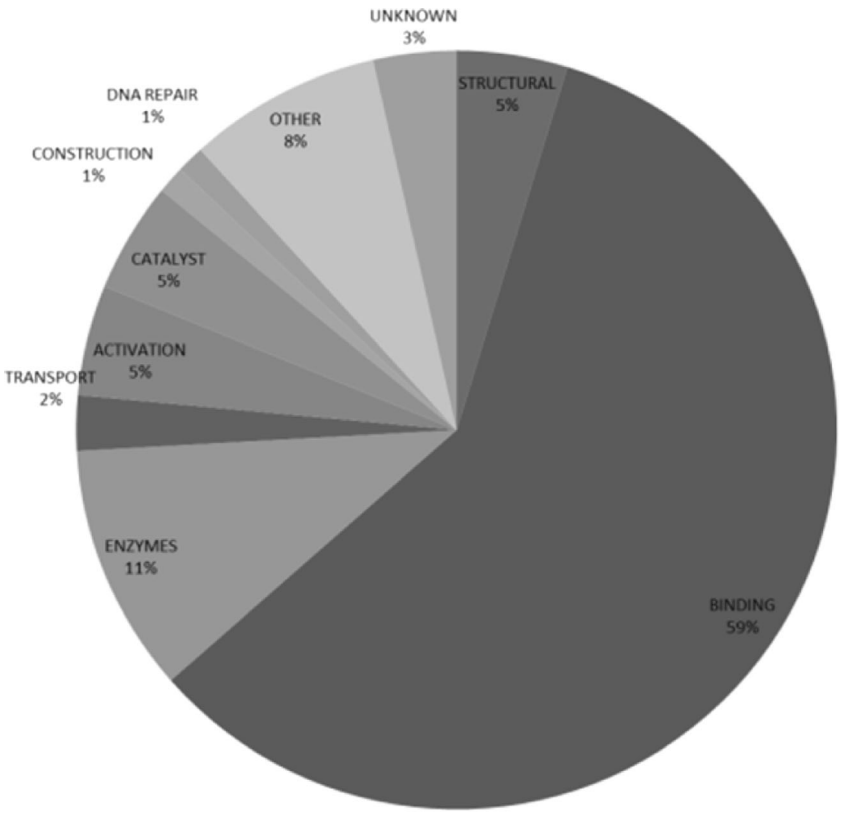


**Fig. 1** Distribution of groups of proteins detected from analysis of cumulus cell samples from blastocyst and non-blastocyst groups

**Table 2** Proteins exclusively detected in cumulus cell samples from the blastocyst and non-blastocyst groups

| Proteins exclusively expressed in the blastocyst group | Proteins exclusively expressed in the non-blastocyst group |
|---|---|
| NADPH/adrenodoxin oxidoreductase | 7-methylguanosine phosphate-specific 5′-nucleotidase |
| Protein AF-17 | Beta-2-glycoprotein 1 |
| Annexin A2 | Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 2 |
| Rho guanine nucleotide exchange factor 1 | Band 3 anion transport protein |
| Rho guanine nucleotide exchange factor 2 | Biogenesis of lysosome-related organelles complex 1 subunit 1 |
| ATP synthase subunit alpha | Cytochrome c oxidase subunit 2 |
| CDK5 and ABL1 enzyme substrate 1 | Protocadherin Fat 2 |
| Cardiomyopathy-associated protein 5 | Histone H2A type 1-B/E |
| Delta(24)-sterol reductase | Histone H3.3C |
| Corticosteroid 11-beta-dehydrogenase isozyme 1 | HEAT repeat-containing protein 1 |
| Alpha-enolase | Hemopexin |
| Endoplasmic reticulum-Golgi intermediate compartment protein 1 | Ig gamma-2 chain C region |
| Alpha-2-HS-glycoprotein | Interleukin enhancer-binding factor 2 |
| Glyceraldehyde-3-phosphate dehydrogenase | Malate dehydrogenase |
| Stress-70 protein | Serine/threonine-protein kinase MRCK gamma |
| 78 kDa glucose-regulated protein | Protein disulfide-isomerase |
| Glutathione S-transferase A1 | Testicular haploid expressed gene protein-like |
| Histone H2A type 1-D | |
| Histone H2A.J | |
| Histone H2AX | |
| Histone H2B type 1-C/E/F/G/I | |
| Heterogeneous nuclear ribonucleoprotein K | |
| Ig heavy chain V-III region TUR | |
| Proto-oncogene serine/threonine-protein kinase mos | |
| Protein disulfide-isomerase A3 | |
| Peptidyl-prolyl cis-trans isomerase B | |
| 40S ribosomal protein S16 | |
| Scavenger receptor class B member 1 | |
| Tubulin beta chain | |
| Serotransferrin | |

**Table 3** Proteins equally and differentially represented in cumulus cell samples from the blastocyst and non-blastocyst groups

| Proteins equally expressed among groups | Proteins differentially expressed among groups |
|---|---|
| ATP-binding cassette sub-family B member 6 | 3 beta-hydroxysteroid dehydrogenase/Delta 5–> 4-isomerase type 2 |
| AP-5 complex subunit zeta-1 | Alpha-1-antitrypsin |
| Protein broad-minded | Alpha-1B-glycoprotein |
| Aromatase | Alpha-1-antichymotrypsin |
| Elongation factor 1-alpha 1 | Actin, aortic smooth muscle |
| Ig alpha-1 chain C region | Actin, cytoplasmic 1 |
| | ADP/ATP translocase 2 |
| | Serum albumin |
| | Angiotensinogen |
| | Annexin A5 |
| | Annexin A6 |
| | Apolipoprotein A-I |
| | N-acetylserotonin O-methyltransferase-like protein |
| | ATP synthase subunit beta |
| | Calreticulin |
| | 60 kDa heat shock protein |
| | Complement C3 |
| | Cholesterol side-chain cleavage enzyme |
| | Endoplasmin |
| | Fibrinogen alpha chain |
| | Fibrinogen beta chain |
| | Fibrinogen gamma chain |
| | Neutral alpha-glucosidase AB |
| | Histone H2B type 1-A |
| | Histone H3.1 t |
| | Histone H4 |
| | Hemoglobin subunit alpha |
| | Hemoglobin subunit beta |
| | Haptoglobin |
| | Ig gamma-1 chain C region |
| | Ig kappa chain C region |
| | Protein disulfide-isomerase A6 |
| | Tubulin alpha-1B chain |
| | Vimentin |

predicting embryo quality and especially for blastocyst formation competence. The identification of patients able to benefit from extended embryo culture programs would be a valuable approach for assisted reproduction success. In fact, although it is known that prolonging the embryo culture period allows for a better selection of embryos for transfer leading to increased implantation rates and reduced risk of twin and higher order pregnancies [38], there is concern that a strategy of blastocyst culture may result in higher cycle cancellation rates [39].

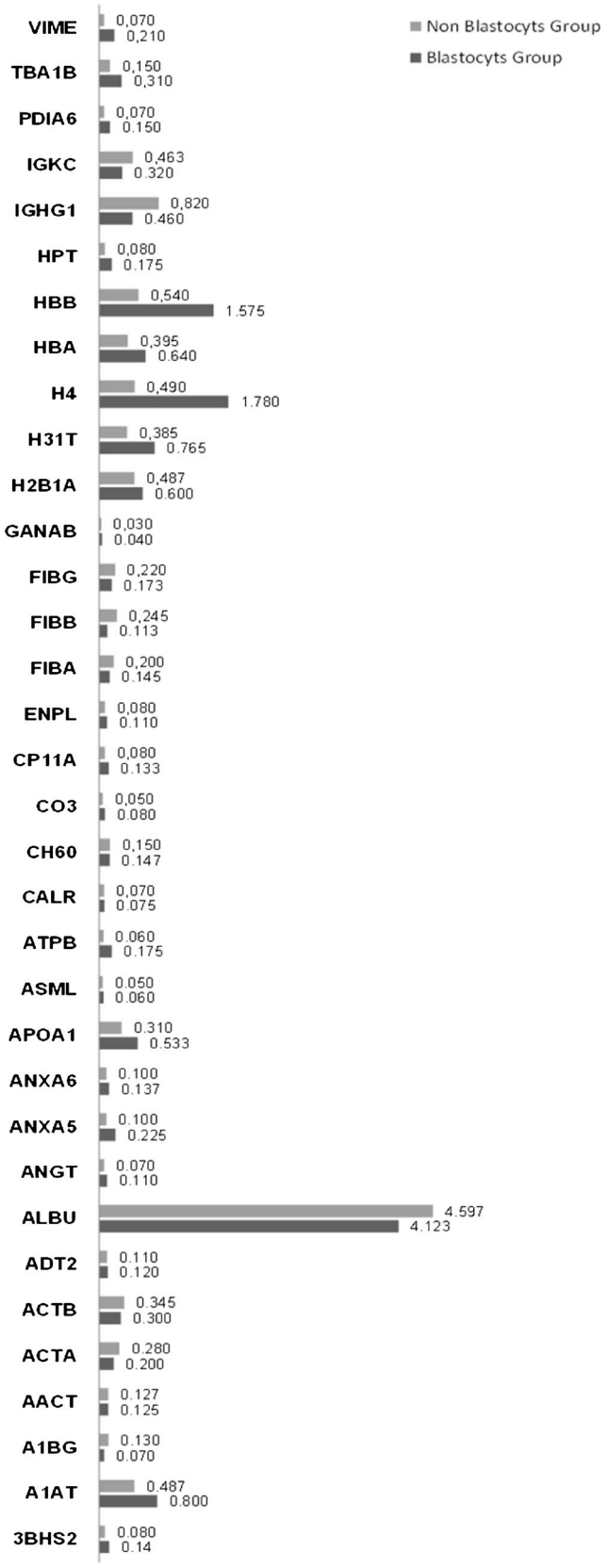


**Fig. 2** Description of signal intensities, expressed as the emPAI value for each differentially expressed in the blastocyst and non-blastocyst groups

Potential CC biomarkers correlated to oocyte quality, embryo competence, or pregnancy outcome have been identified by microarray analysis [14, 24, 26, 40], RT–PCR [41, 42], or quantitative RT–PCR [25, 43, 44]. Although genomics can supply valuable information regarding expected functions, the proteome is the entire complement of proteins expressed by a genome, and therefore, it is more indicative of the phenotype and can provide important information that is complementary to the data obtained from gene expression studies. Moreover, gene expression studies of RNA transcripts do not often predict protein abundance or function [45].

A recently published transcriptomic analysis and meta-analysis of human CCs tried to identify differentially expressed genes and analyze biological processes in human CCs. The authors concluded that transcriptomes of CCs as well as biological functions are distinctive for each cell subpopulation [46]. We also did not find any correlation between the proteins identified here and those from genomic studies. Indeed, changes in gene expression are not necessarily reflected in changes in translated proteins, nor does gene analysis take into account posttranscriptional, translational, or posttranslational changes that relate to cyclical transitions. Of the hundreds of gene expression changes typically identified by microarray, relatively few are common to more than two studies [47, 48]. Comparison of proteomic data with published gene expression data in similar cohorts of women [47, 49, 50] also have revealed an overall lack of correlation between the two, suggesting that posttranscriptional or translational regulation is an important feature in human biology.

Among the proteins identified here, some were exclusively represented in both the blastocyst and the positive pregnancy groups: glutathione S-transferase A4 (GST A4), Ig heavy chain V-III region TUR, protein disulfide-isomerase (PDI) A3, and proto-oncogene serine/threonine-protein kinase mos.

The expression of glutathione S-transferases (GST) in human CCs has already been reported by previous studies [51]. Overexpression of the sub-class GSTA4 has been shown to protect cells from apoptosis [52], and GSTP has also been associated with JNK and protects cells from death signals or oxidative stress [53].

Some GSTs have been shown to be upregulated through the MAPK pathways as self-defense responses to toxins and growth factors [54, 55]. In reproductive cells, p38 MAPK plays a pivotal role in oocyte maturation [56–58] and steroidogenesis [59, 60]. Therefore, it is plausible that GSTs have a strong link to embryo development and implantation.

Immunoglobulins are heterodimeric proteins composed of two heavy (H) and two light (L) chains. They can be separated functionally into variable (V) domains that binds antigens and constant (C) domains that specify effector functions such as activation of complement or binding to Fc receptors [61]. The Ig heavy chain V-III region TUR has already been identified in

**Table 4** Comparison of patients and cycles' characteristics between the positive-pregnancy and negative-pregnancy groups

| Variables | Positive-pregnancy | Negative-pregnancy | *p* value |
|---|---|---|---|
| Maternal age (year) | 33.4.1 ± 3.5 | 34.8 ± 5.8 | 0.456 |
| Paternal age (year) | 36.8 ± 2.1 | 37.3 ± 4.2 | 0.467 |
| FSH administered (IU) | 2145 ± 513 | 2250 ± 435 | 0.669 |
| Estradiol level (pg/mL) | 1598 ± 1348 | 1652 ± 1254 | 0.652 |
| Number of aspirated follicles | 20.3 ± 7.4 | 21.8 ± 9.1 | 0.465 |
| Number of retrieved oocytes | 15.5 ± 8.3 | 14.6 ± 3.8 | 0.562 |
| Number of MII oocytes | 12.3 ± 4.1 | 10,6 ± 5.6 | 0.546 |
| Fertilization rate % | 85.1 ± 10.8 | 83.4 ± 17.3 | 0.256 |

Note: values are mean + SD, unless otherwise noted. NS

the human follicular fluid by LC/MS/MS [62], and it has been hyper-represented in samples from fertilized oocytes [63].

Our results suggest a possibility that these proteins identified in human CCs might not only be involved in folliculogenesis, but also as part of immune response pathways. The importance of the immunologic system during embryo development and reproduction biology is unclear, but a role during embryo implantation had been suggested [64].

Previous reports have identified PDIA3 by MS in the mammalian ovary tissue and [65] in embryo culture media [66]. Its function in the reproductive systems has not yet been elucidated; however, it could be suggested that it includes cell growth and death. The endoplasmic reticulum contains a host of proteins involved in co- and post-translational modifications of newly synthesized polypeptides. The most abundant members of this group of proteins are the PDI [67]. Although

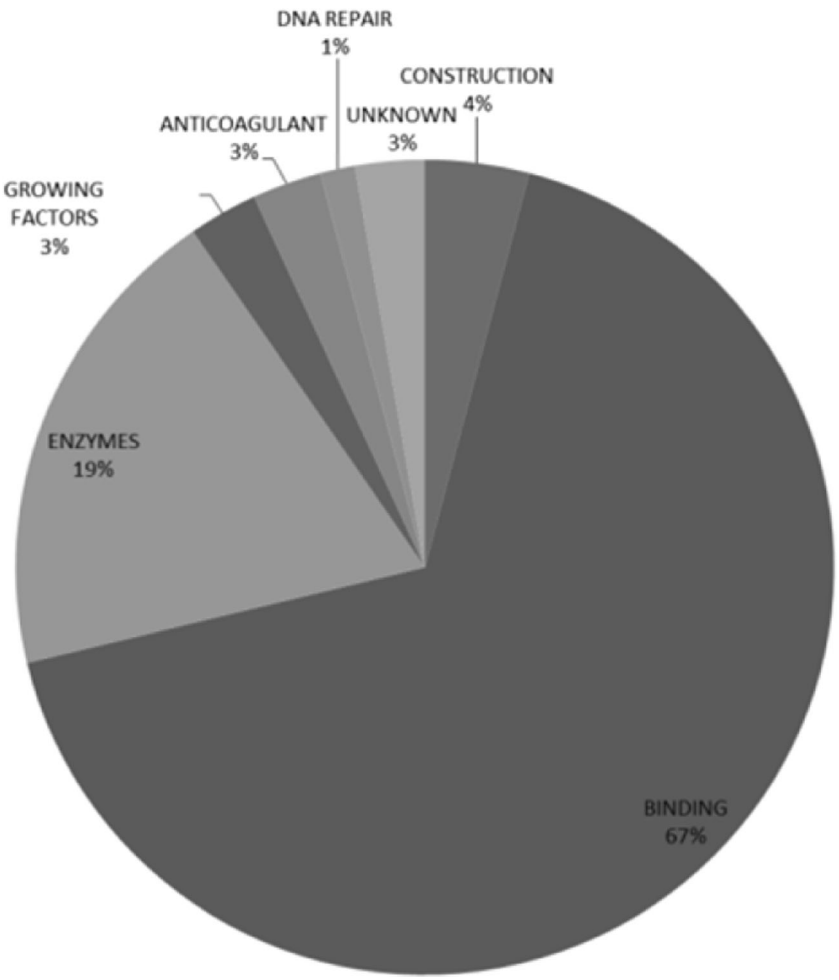


**Fig. 3** Distribution of groups of proteins detected in cumulus cell samples of positive-pregnancy and negative-pregnancy groups

**Table 5** Proteins detected in cumulus cell samples from the positive-pregnancy and negative-pregnancy groups

| Proteins exclusively expressed in the positive-pregnancy group | Proteins exclusively expressed in the negative-pregnancy group |
|---|---|
| ATP-binding cassette sub-family B member 6 | 3 beta-hydroxysteroid dehydrogenase/Delta 5–> 4-isomerase type 2 |
| Actin | Alpha-2-macroglobulin |
| Ceruloplasmin | 2-phosphoxylose phosphatase 1 |
| 60 kDa heat shock protein | Actin |
| Ubiquinone biosynthesis monooxygenase COQ6 | Annexin A2 |
| Carnosine synthase 1 | Clathrin heavy chain 1 |
| Uncharacterized protein C19orf57 | Guanylate-binding protein 4 |
| Uncharacterized protein CXorf66 | Histone H2B type 1-A |
| Elongation factor 1-alpha 1 | Histone H3.3C |
| Alpha-enolase | Heterogeneous nuclear ribonucleoprotein K |
| Protocadherin Fat 2 | Ig kappa chain V-I region Lay |
| Glutathione S-transferase A1 | La-related protein 1 |
| Histone H2A type 2-A | L-lactate dehydrogenase A chain |
| Ig heavy chain V-III region TUR | Nck-associated protein 1 |
| Ig gamma-2 chain C region | Scavenger receptor class B member 1 |
| Immunoglobulin lambda-like polypeptide 5 | Serotransferrin |
| Proto-oncogene serine/threonine-protein kinase mos | |
| Protein disulfide-isomerase A3 | |
| Paired mesoderm homeobox protein 2B | |

it seems clear that proteins contain all the information required for proper folding in the absence of enzyme, PDI may be essential for this process, and it has been suggested that PDI does, in fact, catalyze protein folding within the endoplasmic reticulum [68].

Proto-oncogene serine/threonine-protein kinase mos is essential for the initiation of oocyte maturation [69], for the progression of meiosis I to meiosis II [70], and for the second meiotic metaphase arrest [71]. And together with our findings, these studies suggest that it is also important for oocyte quality, embryo development, and implantation.

It could be argued that the value of a cumulus-based diagnostic test in determining blastocyst formation is limited, as blastocyst formation is determined after in vitro embryo culture, since embryonic gene activation occurs after the second cleavage. However, the oocyte machinery guides the early embryo development and influences in blastocyst formation, and consequently implantation. Considering that the communication between the oocyte and CC is essential for the acquisition of oocyte competence, the proteins identified here may be potential therapeutic targets and may be used, in the future, as supplementation in culture media. Additionally, this study helps to understand the physiology of in vivo maturation in controlled stimulated cycles.

In a previous study [72], the protein profile of individual human embryos was obtained by LC-MS, and it was correlated with morphology; however, the method used in this study resulted in embryo death, limiting or prohibiting its clinical use. In an additional study, Katz-Jaffe et al. [73] non-invasively identified protein biomarkers in the surrounding embryo culture medium and correlated these findings with embryo development. For this study, the protein profile was obtained from pooled samples from thawed embryos cultured to the blastocyst stage. Although it is a promising approach, the analyses of samples obtained from embryos at the blastocyst stage may clutter the use of the obtained information for clinical propose, since embryo transfer is to be performed in a limited time after sample collection.

For this reason, in the present study, the proteomic analysis was performed in CCs rather than in the culture media. Indeed, because of its close contact with the oocyte, CCs reflect the biology and competence of both oocytes and embryos.

An important limitation of this study is the requirement of large amounts of material for proteomic analysis [74]. Indeed, MS requires a large quantity of starting material. This combined with limited template, low protein expression and the lack of sensitivity of proteomic platforms are some of the foremost obstacles [13]. For the present study, the protein concentrations obtained from CCs collected from a single patient were insufficient for LC-MS; therefore, samples from the same group were pooled together in order to achieve the required concentration. For this preliminary study, we aimed to determine which proteins are potential biomarkers for

**Table 6** Proteins equally and differentially represented in cumulus cell samples from the positive-pregnancy and negative-pregnancy groups

| Proteins equally expressed among groups | Proteins differentially expressed among groups |
|---|---|
| Alpha-1-acid glycoprotein 1 | Alpha-1-antitrypsin |
| Alpha-1B-glycoprotein | Alpha-1-antichymotrypsin |
| AP-5 complex subunit zeta-1 | Serum albumin |
| Protein broad-minded | Angiotensinogen |
| Complement C4-A | Annexin A5 |
| Cholesterol side-chain cleavage enzyme | Annexin A6 |
| Endoplasmin | Apolipoprotein A-I |
| Fibrinogen gamma chain | ATP synthase subunit beta |
| Glyceraldehyde-3-phosphate dehydrogenase | Complement C3 |
| Neutral alpha-glucosidase AB | Fibrinogen alpha chain |
| Histone H4 | Fibrinogen beta chain |
| Plasma protease C1 inhibitor | Histone H2A type 1-B/E |
| Ig kappa chain C region | Histone H2B type 1-B |
| Ig lambda-2 chain C regions | Histone H3.1 t |
| Tubulin alpha-1A chain | Hemoglobin subunit alpha |
| Tubulin alpha-1B chain | Hemoglobin subunit beta |
| | Hemopexin |
| | Haptoglobin |
| | Heat shock protein HSP 90-beta |
| | Ig alpha-1 chain C region |
| | Ig gamma-1 chain C region |
| | Peptidyl-prolyl cis-trans isomerase B |

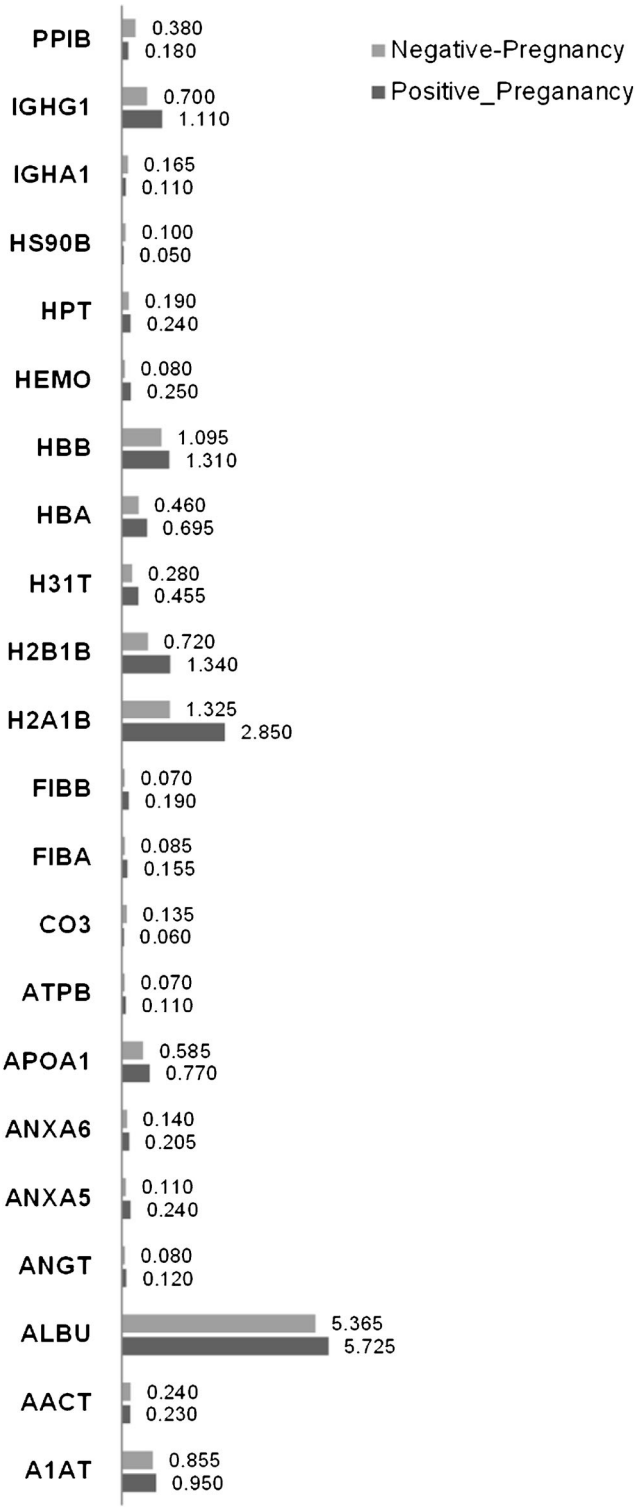


**Fig. 4** Description of signal intensities, expressed as the emPAI value for each differentially expressed in the positive-pregnancy and negative-pregnancy groups.

blastocyst formation and pregnancy. Our results provide a rationale for conducting further research aimed to determine evaluating diagnostic and to therapeutic targets for oocyte in vitro maturation and embryo culture.

Although a different protein profile could be identified among patients in different groups, it is of paramount importance to develop a technique to identify and to differentiate the protein profile of a single patient's sample or even from CCs of a single complex cumulus oophorus.

Although the LC/MS technique has emerged with promising results in the omics field, it is not able to detect the proteins that are really expressed in cells due to some limitations (peptide mass, efficiency of digestion, volatility, LC resolution etc.); therefore, the data described here are limited to protein detection technique by LC/MC approach and protein abundance in CCs.

In the present study, potential biomarkers for blastocyst formation potential and pregnancy outcome were identified. The next step is to individually identify these proteins and to determine their frequency in subjects. Therefore, CC proteomics may be useful for the prediction of pregnancy success and the identification of patients that should be included in extended embryo culture programs or patients who would benefit from cleavage stage embryo transfers.

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