Expressed proteins and activated pathways in conditioned embryo culture media from IVF patients are diverse according to infertility factors

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ABSTRACT

Objective: Objective: Given that the embryo culture medium secretome reflects the embryo development, we hypothesize that protein profiles are affected according to infertility factors, which can be responsible for detrimental embryonic developmental competence. The aim of this study was to screen the protein profile of conditioned embryo culture media in patients presenting deep infiltrating endometriosis (ENDO) and polycystic ovarian syndrome (PCOS) undergoing IVF, by proteomics approaches. The control group was constituted by tubal factor patients.

Methods: Patients underwent in vitro fertilization (IVF) treatment as routine and oocytes were fertilized by ICSI. The embryos were group cultured until day 3 of development, and after transfer the culture media were collected. For the proteomics analysis, two pools of samples were prepared for groups CONTROL and PCOS, and 4 pools of samples for group DIE. Samples were prepared to deplete high abundant proteins and followed evaluated by high throughput proteomics approach.

Results: The embryonic organ and tissue development were physiological functions activated, based on proteins identified in the 3 study groups of samples. The samples coming from DIE patients presented a high calcium activity and on the other hand, embryos coming from PCOS patients showed a decreased calcium action. Other pathways as grow factors through the EGF signaling pathway overexpressed in ENDO culture medium and protein kinase A in PCOS were also observed.

Conclusions: Proteomic embryonic secretome will advance our knowledge of early embryogenesis and additionally could lead to improved selection of embryos for transfer warrants further investigation.

Keywords: embryo, conditioned culture media, secretome, IVF, endometriosis, ovarian polycystic ovarian syndrome

INTRODUCTION

In vitro fertilization (IVF) has gained attention since its introduction in 1978 and, to date, more than 2 million babies have been born worldwide through assisted reproductive technologies (ART). Developments in technologies for IVF, embryo culture and therapies for ovarian stimulation make ART relatively successful. However, it has its limitations with >50% of IVF embryos failing to implant. Selecting viable embryos for transfer is a key factor for the success of IVF treatment; and requires accurate pre-transfer assessment of embryo viability and morphology. However, the available methods to identify high implantation potential embryos are still limited (Gardner et al., 2015).

The field of human ART would therefore benefit from more quantitative methods of determining embryo viability and implantation potential to further improve the pregnancy rates. It is known that soluble ligands and its receptors mediate human pre-implantation embryo development and implantation (Thouas et al., 2015). Non-invasive analyses of the embryonic secretome, including proteins secreted by the embryo into the surrounding media, provide an alternative method for assessing an embryo’s viability. These results may promote an understanding of the biological mechanisms, and potentially allow for the success of IVF developmental biomarkers.

A study evaluating conditioned media from non-manipulated human embryos cultured in vitro demonstrated that it contains extracellular vesicles and bearing the traditional microvesicle and exosome marker proteins CD63, CD9 and ALIX, which may suggested a way of communication at the maternal-fetal interface (Giacominini et al., 2017). A number of researchers has used the proteomics approach related to human reproduction in several subareas as ectopic pregnancy (Gerton et al., 2004), miscarriage (Liu et al., 2006), follicular fluid (Lo Turco et al., 2010; Kim et al., 2006; Silberstein et al., 2009; Estes et al., 2009; Twigt et al., 2012; Liu et al., 2007), oocytes (Ferreira et al., 2010), endometrium (Matorras et al., 2018) and embryos (Ferreira et al., 2010; Katz-Jaffe & Gardner, 2007; Katz-Jaffe et al., 2009; Domínguez et al., 2009). Recently, various proteins have been investigated as an embryo biomarker in the spent culture media (Butler et al., 2013; Mains et al., 2011; Ziebe et al., 2013; Cortezzi et al., 2013; Domínguez et al., 2008) given that the embryo culture medium secretome reflects the embryo development. We hypothesize that protein profiles are affected according with infertility factors, which can be responsible for detrimental embryonic developmental competence, ensuing on lower IVF success rates. Hence, we studied samples from
patients presenting deep infiltrating endometriosis (ENDO) and polycystic ovarian syndrome (PCOS), which are the most frequent female factors of infertility. Endometriosis is present in up to 50% (Missmer & Cramer, 2003) of infertile women and PCOS is present in the main cause of anovulatory infertility (Thessaoniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2008). It is clear that the ovarian microenvironment is affected by the presence of endometriosis (Regiani et al., 2015; Singh et al., 2013; Karita et al., 2011) and PCOS (Huang et al., 2013; Ambekar et al., 2015; Roth et al., 2014).

Based on the knowledge that the potential of embryo development is an extension of oocyte quality, and the last is directly influenced by the microenvironment of ovary, possibly being affected by the presence of PCOS or END0, we hypothesized the embryo secretoma during its pre-implantation development could also vary reflecting the infertility factor condition. The aim of this study was to screen the protein profile of conditioned embryo culture media in patients with PCOS and END0 undergoing IVF by proteomics approaches.

MATERIAL AND METHODS

Sampling
It was included in the study conditioned embryo culture media samples obtained from patients submitted to IVF cycle at the Huntington - Reproductive Medicine, Sao Paulo - Brazil. Institutional Ethical approval secured for the use of samples for the purpose of this research and the samples were donated by consenting patients whose signed the Informed Consenting Form as established by ethics for assisted reproduction treatment and research (CFM, 2013). Clinical characteristics and outcomes were obtained from patients' charts.

All women presented with the following inclusion criteria: infertile patients undergoing ICSI cycles with ejaculated sperm, presence of both ovaries, regular menstrual cycle, body mass index (BMI) lower than 35 Kg/m², no ongoing infectious disease, no uterus pathology, basal follicular stimulating hormone (FSH) measurement <14IU/L, and basal estradiol measurement <70pg/mL. The exclusion criteria were presence of gynecological bleeding, hydroalpinx, allergy to gonadotropins or other medications used in the treatment, abusive use of any other medications during treatment, and male partners presenting with severe oligozoospermia.

Among patients included, three groups were analyzed according to infertility etiology: tubal factor patients who were considered the control group for this study (CONTROL; n=6), infertile patients presenting PCOS (PCOS; n=7) and infertile patients presenting endometriosis grades III and IV (ENDO; n=14). The diagnosis of infertility was carried out according to international patterns.

Patients were submitted to pituitary blockage and controlled ovarian stimulation as routine. The pituitary blockage was obtained with a GnRH agonist (Lupron kit, Abbot SA Societe Française des Laboratoires, France), and the ovarian stimulation was performed using recombinant FSH (rFSH, Gonal-F® Serono, Switzerland). When at least two follicles reached a diameter of 16 mm, the final follicular maturation was triggered with 250µg of recombinant hCG (rHCG, Ovidrel®, Serono, Switzerland). Oocyte retrieval was performed after 35 to 36 hours by transvaginal ultrasound-guided aspiration, and the luteal phase was supported by 90mg of daily progesterone (Crinone®, Serono, Switzerland) via vaginal approach.

After oocyte recovery and denudation, all of the mature oocytes were fertilized by ICSI (Palermo et al., 1992) as per the routine of the clinic. The normally fertilized oocytes were identified and cultured in groups until day 3 (D3) in 1 ml of cell culture medium (G-1 Plus, Vitrolife) under a layer of paraffin oil (OVOIL, Vitrolife), in incubators with 5% O₂ and 5% CO₂. From D3 until the blastocyst stage (D5), the embryos were cultured in 1 ml of medium containing 10% human albumin (CSCM, Irvine Scientific) under a layer of paraffin oil in triple gas incubators (90% N₂, 5% O₂, and 5% CO₂). The blastocysts were morphologically classified and the highest grades ones were selected for transfer to the women's uterus using a catheter guided by ultrasound. The culture media was collected on day 3 of development and stored at -80°C until use. For the proteomics analysis, two pools of samples were prepared for groups CONTROL and PCOS, and four pools for group END0.

MS analysis
All analyzes in this step of study were carried out at Proteomics and Mass Spectrometry Facility, Center for Drug Discovery and Innovation (CDDI), University of South Florida (USF), Tampa - FL, USA. Arbitrary samples were used for methods standardization, and clinical samples were pooled into 3 to 4 samples pools according to pre-established groups (Control, PCOS and END0).

Samples were prepared by using two steps of purification in order to eliminate the excess of albumin. First, samples were submitted to microfiltration using a 30KDa pore filter (Amicon Ultra-0.5, Centrifugal Filter Devices - 30 KDa, Millipore, USA) and the filtrated material was collected. The material retained in the filter (higher than 30 KDa) was albumin depleted using affinity chromatography columns (Albumin & IgG Depletion SpinTrap, GE Healthcare Life Sciences, USA) according to manufacturer's protocol. The two aliquots of each sample were joined and protein concentrations were measured by Bradford method (Pierce 660nm Protein Assay Reagent, Thermo Scientific), using pre-diluted albumin standard curve (Pre-Diluted Protein Assay Standards: Bovine Serum Albumin, Thermo Scientific) and the absorbance's were acquired at 660 nm. Two hundred and fifty micrograms (250µg) of protein for each sample were diluted in 8M urea buffer and digested using FASP kits (Expeodeon, Inc, USA) as per vendor specifications. Tryptic peptides were acidified by adding trifluoroacetic acid (TFA 1%) to get the 0.1% of TFA concentration, and then desalted on solid phase columns (DSC-18, Solid Phase Extraction - SPE, Discovery DSC-18 SPE Products, Sigma Aldrich). Samples were vacuum dried (Wisniewski et al., 2009).

Tryptic peptides resulting from the preparation were fractionated by liquid chromatography mass spectrometry (HPLC-MS/MS). Five microliters injection of each sample loaded with aqueous solvent (0.1% formic acid in water) were separated by nano-flow reversed phase HPLC using a Nano-LC Ultra 2D+ (Eksigent, Dublin, CA) equipped with a Proteopep 2 Integra Fit trapping column (100 µm i.d. x 2.5 cm; C18, 5 µm, 300Å) and a Proteopep 2 Integra Fit analytical column (75 µm i.d. x 10 cm; C18, 5 µm, 300Å, New Objective, Woburn, MA). Samples (0.5 to 3 µg in 5 µL) were loaded onto the trap column at 2 µL/min (Solvent A) for 12 minutes, after which a valve was switched to include the analytical column. Peptides were then eluted with a gradient (300 nL/min) of 2% B to 35% B over 240 minutes (Solvent A: 100% H₂O, 0.1% formic acid, Solvent B: 98% acetonitrile, 0.5% formic acid). Eluates were delivered by electrospray ionization (ESI) at 2 kV and analyzed by data-dependent MS/MS on a LTQ XL (Thermo Scientific, San Jose, CA, USA) mass spectrometer equipped with XCalibur (version 2.0.7) data acquisition software. Full MS scans were set for centroid mode at normal resolution. MS/MS scans were performed on the top ten most intense ions from each full scan to acquire spectral data for peptide identification. Dynamic exclusion durations were set to 180s with one repeat and a list size of 500.
Raw data acquired were searched against the European Bioinformatics Institute’s (EBI) universal protein resource database (UniProt, November 2014) using Mascot (version 2.2). Peptide and protein validations were performed using the Scaffold platform (version 3.00.08). Search parameters used were as follows: precursor mass error tolerance of 20 ppm; fragment mass error tolerance of 0.1 Da; trypsin as a protease with one missed cleavage allowed; and carbamidomethylation of cysteine as fixed modification and oxidation of methionine as a variable modification. Identified peptides and proteins were validated and visualized with Scaffold 3.6 (Proteome Software, Portland, OR).

The proteins identified in the previous analysis were analysed using Ingenuity® Pathway Analysis software (IPA®, Qiagen, Redwood, USA). We considered peptide threshold of 20% and the biological processes were staged according to the IPA® Knowledge Base. The association between the identified proteins and canonical pathways of the database was also accessed with IPA® software using Fisher’s exact test (significance of \( p < 0.01 \)). From 2880 proteins identified, decoy proteins that are considered false identification were excluded. Two sets of analysis were carried out with remaining proteins using the IPA®. First of all we identified proteins exclusive in each study group (PCOS or ENDO) compared with CONTROL. We considered exclusively expressed those protein expressed in at least two sample pools of each group and absent on the other comparison groups. Then, we evaluated proteins differentially expressed in the study groups (PCOS or ENDO) compared with CONTROL and considered fold change higher than two.

RESULTS

Table 1 describes the demographic data regards the patients included in this study. In the ENDO group, 17 proteins were exclusively expressed (present in at least two of four samples pools), and two were over expressed compared to CONTROL (Table 2). The canonical pathways identified which were related to proteins exclusively expressed in ENDO group were associated with calcium metabolism (calcium signaling \( p = 0.0057 \) and transport \( p = 0.0058 \)) and calcium induced T-lymphocyte apoptosis \( p = 0.041 \)) and EGF signaling \( p = 0.035 \) (Figure 1).

On the other hand, in the PCOS group presented 284 proteins exclusively expressed and one overexpressed with fold change higher than 2 compared to CONTROL, which were associated with the following pathways: Protein Kinase A signaling and calcium signaling were downregulated, and GADD45 signaling, hydrocarbon receptor signaling and GDP-L-fucose biosynthesis II were upregulated (Figure 2). The PCOS group had two pools of samples analysed and from 284 proteins exclusively expressed in the PCOS group, six were present in both pools of samples (Table 3).

Based on proteins identified, the CONTROL group had the following cellular and molecular function highlighted: cellular development, cellular movement, amino acid metabolism, small molecule biochemistry, cellular assembly and organization, which were function associated to general cellular development. Also, the embryonic organ and tissue development were physiological functions activated based on proteins identified in the 3 study groups of samples.

DISCUSSION

In the last decade, the application of proteomics high throughput methodologies to human reproductive fluids and cells have delineated novel biochemical functional profiles and molecular processes that characterize and may affect folliculogenesis, oocyte maturation and quality, and as consequence the embryo development potential and clinical outcomes. Embryos are programmed to produce soluble ligands and receptors, which elicit changes in embryo developmental phenotype and also modulating local responses in the receptive endometrium determining an embryo maternal cross talk during peri-implantational period. These signaling pathways are highly complex and it understanding has been gradually improved (Thouas et al., 2015). The embryos from in vitro fertilization also produce soluble factors that are secreted in the spent culture media. Proteomics in spent culture media involved the measurement of amino acids (Brison et al., 2004; Sturmy et al., 2008), proteins (Katz-Jaffe et al., 2006a;b; 2009; Nyalwiide et al., 2013) and metabolomics evaluates how the embryo alters its microenvironment (Scott et al., 2008; Leese et al., 2008). Authors have been trying to find if changes in the levels of some molecules are associated with implantation potential of embryos and clinical outcomes of IVF cycles.

Different approaches were necessary based on the nature of the specimens and the types of analyses being done. The standardization of proteomics approaches embryo culture medium was developed for this study, there we could obtain data available to a general protein profile in the samples. Depletion of abundant proteins combined with multidimensional protein fractionation was instrumental in allowing the study of middle- and lower-abundance proteins. Our study demonstrates that the technology can provide a consistent result given the restriction of starting material and time to analysis. These proteins with their regulatory pathways may play a vital role in reproductive process.

In embryos derived from ENDO group, we observed over expression of EGF signaling pathway compared to CONTROL. EGF is a growth factor family which has been already identified to be expressed by the human preimplantation embryo (Chia et al., 1995) and in vitro studies also showed the EGF treatment increases early human embryo development and blastocyst formation (Yu et al., 2012). Animal studies have shown that EGF enhanced developmental competence of cat embryos by stimulating cell proliferation and modulating the EGFR expression at...
On the other hand, an in vitro study cultured mice embryos with human tubal fluid from endometriosis patients and observed that the levels of embryonic EGF, IGF-I, and their receptors were increased, and it attenuated embryo development by impairing embryonic growth factor/receptor/signal transduction (Ding et al., 2010). Endometriosis has been associated with impaired IVF outcomes (Harb et al., 2013; Somigliana & Garcia-Velasco, 2015). Based on that, we can speculate the higher expression of EGF in endometriosis group may have an ovarian origin and it can be associated with the mechanism of embryo development of those patients. Other pathways highlighted were associated with calcium signaling. Studies have shown that mitochondria has an enormous capacity to regulate Ca^{2+} (Giacomello et al., 2007). The embryo development is strongly correlated with the activity of mitochondria, and the abnormal

**Table 2.** Proteins exclusively expressed and over expressed in the ENDO compared to CONTROL group

| Group ENDO compared to CONTROL | Protein name |
|--------------------------------|--------------|
| Exclusive                      | GIGYF2 protein OS=Homo sapiens GN=GIGYF2 PE=2 SV=1 | A6H8W4_HUMAN (+5) 152 98% 45.2 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=DAT PE=4 SV=1 | E7RU0_HUMAN (+6) 616 74% 46.0 |
| Exclusive                      | Isoform 2 of U4/U6 small nuclear ribonucleoprotein Prpr4 OS=Homo sapiens GN=PRPF4 | sp|Q43172-2|PRPF4_HUMAN (+1) 58 76% 45.0 |
| Exclusive                      | C1q-related factor OS=Homo sapiens GN=C1QL1 PE=2 SV=1 | C1QRF_HUMAN 26 74% 40.7 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=MFF PE=4 SV=1 | C9HFS_HUMAN 16 55% 45.2 |
| Exclusive                      | Isoform 2 of Neuron navigator 1 OS=Homo sapiens GN=NAV1 | sp|Q8NEY1-2|NAV1_HUMAN (+4) 202 57% 41.1 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=ATP2C1 PE=4 SV=1 | D6REJ1_HUMAN (+6) 12 54% 43.8 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=MEN1 PE=4 SV=1 | E7EN32_HUMAN (+3) 61 82% 44.8 |
| Exclusive                      | Olfactory receptor 6B2 OS=Homo sapiens GN=OR6B2 PE=2 SV=2 | OR6B2_HUMAN 35 65% 46.3 |
| Exclusive                      | Angiopoietin-related protein 6 OS=Homo sapiens GN=ANGPTL6 PE=1 SV=1 | ANGL6_HUMAN 52 57% 45.3 |
| Exclusive                      | Arginine-glutamic acid dipeptide repeats protein OS=Homo sapiens GN=RERE PE=1 SV=2 | sp|Q9P2R6|RERE_HUMAN 172 54% 43.7 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=ADAR PE=4 SV=1 | E7ENU4_HUMAN (+5) 141 97% 45.2 |
| Exclusive                      | Inositol 1,4,5-trisphosphate receptor type 2 OS=Homo sapiens GN=ITPR2 PE=1 SV=2 | sp|Q14571|ITPR2_HUMAN 308 64% 44.9 |
| Exclusive                      | Isoform 2 of Transcriptional-regulating factor 1 OS=Homo sapiens GN=TRERF1 | sp|Q96PN7-2|TRERF1_HUMAN (+3) 106 97% 44.7 |
| Exclusive                      | Isoform 2 of Ubiquitin-conjugating enzyme E2 W OS=Homo sapiens GN=UBE2W | UBE2W_HUMAN 19 93% 45.0 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=LAMB2 PE=4 SV=1 | E7EMH6_HUMAN 40 79% 44.0 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=MDM1 PE=4 SV=1 | E7EPQ3_HUMAN (+2) 77 84% 44.7 |
| Over expressed (FC=2.90;p=0.601) | Uncharacterized protein OS=Homo sapiens GN=BRCA1 PE=4 SV=1 | E7EMPO_HUMAN (+9) 181 68% 45.4 |
| Over expressed (FC=2.31;p=0.483) | Histone H1.4 OS=Homo sapiens GN=HIST1H1E PE=1 SV=2 | H14_HUMAN 22 88% 45.8 |

FC=Fold Change. KDa=kilodaltons
distribution of mitochondria exert negative effects on
the embryogenesis due to the abnormal ATP distribution
(Nagai et al., 2006), because high energy supply around
nucleus is very important during embryonic development
(Wang et al., 2009), and may perform an important func-
tion in embryonic cell-cycle transition and embryonic axis
establishment (Whitaker, 2008). A study as shown that
conditioned medium from human embryos also trigger
calcium oscillations in human endometrial epithelial cells,
and importantly, the endometrial responses are affected
by developmentally competency of embryos as low-quality
human embryos trigger prolonged and disorganized
calcium oscillations, leading to a uterine stress response
(Brosens et al., 2014). In our study, while samples coming
from endometriosis patients presented calcium-signaling
upregulated, the PCOS samples showed it downregulated.
In spite of most of publication find similar clinical outcomes
after IVF in PCOS patients, it is related to alterations in
oocyte quality and consequently in embryo quality, may be
due to endocrine and intra-ovarian environment (Sermon-
dade et al., 2013). The calcium metabolism downregulated
in the PCOS embryos might reflect the poorer oocyte qual-
ity observed in those patients.
Protein kinase A signaling is also down regulated in
PCOS samples. A study in non-human primate showed
that mechanisms underlying adrenal hyperandrogenism
seen in the human condition of PCOS are further considered in terms of the effects of altered relative expression of CYP17, HSD3B2 and CYB5, as well as the altered signaling responses of various kinases including protein kinase A (Abbott & Bird, 2009). GADD45, another protein over expressed in PCOS samples, is a protein that is often induced by DNA damage and other stress signals associated with growth arrest and apoptosis (Salvador et al., 2013).

The data on secretory activity can vary considerably suggesting that embryo can intrinsically modulate the microenvironment and may reflect developmental plasticity rather than quality. In vitro studies provided evidence for a putative mechanism by which the decidualized stromal cells sense developmental embryos through the molecules secreted in the culture media (Brosens et al., 2014). Also, signals emanating from competent human embryos triggered a very specific transcriptional response in the mouse uterus, characterized by the induction of multiple metabolic genes (Teklenburg et al, 2010). Hence, focusing on exposures that infertility factors per se influence differential proteome profiles in the embryo culture media, can help understand its effects in the embryo developmental plasticity and competence, and consequently in IVF outcomes.

This study relies on the proteomic profile of conditioned embryo culture media in different infertility conditions. We did not correlated the proteins identified with the embryo characteristics as samples come from embryos cultured in groups and they were group cultured and we joined them into pools according to infertility factors to be analyzed. Also, the sample size is reduced and outcomes should be confirmed using immunodetection technique and in a higher number of samples. Due to high concentration of contaminants in the culture media, as albumin, samples were submitted to a number of process which might depleted other less abundant proteins. To date, the proteomic approach has proven to be a challenging task due to the complexity and diversity of the human embryo and heterogeneity across patients and within embryo cohorts.

In summary, the embryonic, organ and tissue development were physiological functions activated based on proteins identified in the three study groups of samples. The embryos coming from endometriosis patients present a high calcium activity and on the other hand, embryos coming from PCOS patients showed a decreased calcium action, which may be related to embryo developmental competence or plasticity. Other pathways as growth factors action, which may be related to embryo developmental competence or plasticity. Other pathways as growth factors through the EGF signaling pathway overexpressed in endometriosis embryos and protein kinase A in PCOS were also observed. Characterizing the proteomic embryonic secretome will advance our knowledge of early embryogenesis and the embryo’s role during the initial stages of implantation. Additionally, the activity in embryo culture medium could lead to improved selection of embryos for transfer warrants further investigation.

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**Table 3. Proteins exclusively expressed and over expressed in the PCOS compared to CONTROL group**

| Group PCOS compared to CONTROL | Protein name | Accession Number | Molecular Weight (KDa) | Protein identification probability | Mascot identity score |
|--------------------------------|--------------|------------------|------------------------|----------------------------------|----------------------|
| Exclusive                      | SHANK2 protein OS=Homo sapiens GN=SHANK2 PE=2 SV=1 | B7ZKU9_HUMAN (+3) | 29                     | 64%                              | 41.4                 |
| Exclusive                      | Uncharacterized protein OS=Homo sapiens GN=HIVEP1 PE=4 SV=1 | F5H212_HUMAN (+3) | 62                     | 73%                              | 44.0                 |
| Exclusive                      | Putative uncharacterized protein DKFZp434N071 OS=Homo sapiens GN=DKFZ-p434N071 PE=2 SV=2 | Q68CU6_HUMAN (+1) | 58                     | 57%                              | 40.0                 |
| Exclusive                      | Isoform 2 of Protein strawberry notch homolog 1 OS=Homo sapiens GN=SNBO1 | sp|A3KN83-2|SB-NO1_HUMAN (+2) | 154 | 72% | 41.6 |
| Exclusive                      | Collagen alpha-1(III) chain OS=Homo sapiens GN=CO-L3A1 PE=1 SV=4 | sp|P02461|-CO3A1_HUMAN | 139 | 73% | 41.5 |
| Exclusive                      | Isoform 2 of Phosphofurin acidic cluster sorting protein 1 OS=Homo sapiens GN=PACS1 | sp|Q6VY07-2|PACS1_HUMAN (+1) | 109 | 69% | 43.8 |
| Over expressed (FC=2.91; p=0.598) | Isoform 3 of Cytosolic carboxypeptidase 1 OS=Homo sapiens GN=AGTPBP1 | CBPC1_HUMAN | 139 | 55% | 41.6 |

FC=Fold Change. KDa=kilodaltons.
CONFLICT OF INTERESTS
The authors have no conflict of interest to declare.

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REFERENCES
Abbott DH, Bird IM. Nonhuman primates as models for human adrenal androgen production: function and dys- function. Rev Endocr Metab Disord. 2009;10:33-42. PMID: 18683055 DOI: 10.1007/s11154-008-9099-8

Ambekar AS, Kelkar DS, Pinto SM, Sharma R, Hinduja I, Zaveri K, Pandey A, Prasad TS, Gowda H, Mukherjee S. Proteomics of follicular fluid from women with polycystic ovary syndrome suggests molecular defects in follicular development. J Clin Endocrinol Metab. 2015;100:744-53. PMID: 25395639 DOI: 10.1210/jc.2014-2086

Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkweed J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod. 2004;19:2319-24. PMID: 15298971 DOI: 10.1093/humrep/deh409

Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, Steel JH, Christian M, Chan YW, Boomsma CM, Moore JD, Hartshorne GM, Sućurović S, Mulac-Jericevic B, Heijnen CJ, Quenby S, Koerkamp MJ, Holstege FC, Shmygol A, Macklon NS. Uterine selection of human embryos at implantation. Sci Rep. 2014;4:3894. PMID: 24503642 DOI: 10.1038/srep03894

Butler SA, Luttoo J, Freire MO, Abban TK, Borrelli PT, Iles RK. Human chorionic gonadotropin (hCG) in the secretome of cultured embryos: hyperglycosylated hCG and hCG-free beta subunit are potential markers for infertility manage- ment and treatment. Reprod Sci. 2013;20:1038-45. PMID: 23439616 DOI: 10.1177/1933719112472739

CFM-Conselho Federal de Medicina. Resolução CFM No 2.013/2013. Normas éticas para a utilização das técni- cas de reprodução assistida. Brasília: CFM; 2013. Available at: http://www.portalmedico.org.br/resolucoes/cfm/2013/2013_2013.pdf

Chia CM, Winston RM, Handyside AH. EGF, TGF-alpha and EGFR expression in human preimplantation embryos. Devel- opment. 1995;121:299-307. PMID: 7768173

Cortezzi SS, Cabral EC, Trevisan MG, Ferreira CR, Setti AS, Braga DP, Figueira Rde C, Iaconelli A Jr, Eberlin MN, Borges E Jr. Prediction of embryo implantation potential by mass spectrometry fingerprinting of the culture medium. Reproduction. 2013;145:453-62. PMID: 23404850 DOI: 10.1530/REP-12-0168

Ding GL, Chen XJ, Luo Q, Dong MY, Wang N, Huang HF. Attenuated oocyte fertilization and embryo develop- ment associated with altered growth factor/signal trans- duction induced by endometriotic peritoneal fluid. Fer- til Steril. 2010;93:2538-44. PMID: 20045520 DOI: 10.1016/j.fertnstert.2009.11.011

Dominguez F, Gadea B, Esteban FJ, Horcajadas JA, Pel- licer A, Simón C. Comparative protein-profile analysis of implanted versus non-implanted human blastocysts. Hum Reprod. 2008;23:1993-2000. PMID: 18556682 DOI: 10.1093/humrep/den205

Dominguez F, Pellicer A, Simón C. The human embryo proteome. Reprod Sci. 2009;16:188-90. PMID: 19087971 DOI: 10.1177/1933719108328612

Estes SJ, Ye B, Qiu W, Cramer D, Hornstein MD, Missmer SA. A proteomic analysis of IVF follicular fluid in women <or=32 years old. Fertil Steril. 2009;92:1569-78. PMID: 19890758 DOI: 10.1016/j.fertnstert.2008.08.120

Ferreira CR, Saraiva SA, Catharino RR, Garcia JS, Gozz- zo FC, Sanvido GB, Santos LF, Lo Turco EG, Pontes JH, Basso AC, Bertolla RP, Sartori R, Guardheiro MM, Perecin F, Meirelles FV, Sangalli JR, Eberlin MN. Single embryo and oocyte lipid fingerprinting by mass spectrometry. J Lipid Res. 2010;51:1218-27. PMID: 19965589 DOI: 10.1194/jlr.D001768

Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. Hum Reprod Update. 2015;21:727-47. PMID: 25567750 DOI: 10.1093/humupd/dmu064

Gerton GL, Fan XJ, Chittams J, Sammel M, Hummel A, Strauss JF, Barnhart K. A serum proteomics approach to the diagnosis of ectopic pregnancy. Ann N Y Acad Sci. 2004;1022:306-16. PMID: 15251976 DOI: 10.1196/annals.1318.046

Giacomelli M, Drago I, Pizzo P, Pozzan T. Mitochondri- al Ca2+ as a key regulator of cell life and death. Cell Death Differ. 2007;14:1267-74. PMID: 17431419 DOI: 10.1038/sj.cdd.4402147

Giacomini E, Vago R, Sanchez AM, Podini P, Zarovni N, Murdica V, Rizzo R, Bortolotti D, Candiani M, Viganò P. Secretome of in vitro cultured human embryos contains extracellular vesicles that are uptaken by the mater- nal side. Sci Rep. 2017;7:5210. PMID: 28701751 DOI: 10.1186/s41598-017-05549-w

Harb HM, Gallos ID, Chu J, Harb M, Coomarasamy A. The effect of endometriosis on in vitro fertilisation outcome: a sys- tematic review and meta-analysis. BJOG. 2013;120:1308-20. PMID: 23834505 DOI: 10.1111/1471-0528.12366

Huang X, Hao C, Shen X, Liu X, Shan Y, Zhang Y, Chen L. Differences in the transcriptional profiles of human cumulus cells isolated from MI and MII oocytes of patients with polycystic ovary syndrome. Reproduction. 2013;145:597-608. PMID: 23603633 DOI: 10.1530/REP-13-0005

Karita M, Yamashita Y, Hayashi A, Yoshida Y, Hayashi M, Yamamoto H, Tanabe A, Terai Y, Ohmichi M. Does advanced-stage endometriosis affect the gene expression of estrogen and progesterone receptors in granulosa cells? Fertil Steril. 2011;95:889-94. PMID: 21269613 DOI: 10.1016/j.fertnstert.2010.12.026

Katz-Jaffe MG, Gardner DK, Schoolcraft WB. Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability. Fertil Steril. 2006a;85:101-7. PMID: 16412738 DOI: 10.1016/j.fertnstert.2005.09.011
Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. Fertil Steril. 2006b;86:678-85. PMID: 16952510 DOI: 10.1016/j.fertnstert.2006.05.022

Katz-Jaffe MG, Gardner DK. Embryology in the era of proteomics. Theriogenology. 2007;68:S125-30. PMID: 17477967 DOI: 10.1016/j.theriogenology.2007.03.014

Kim YS, Kim MS, Lee SH, Choi BC, Lim JM,Cha KY, Baek KH. Proteomic analysis of recurrent spontaneous abortion: Identification of an inadequately expressed set of proteins in human follicular fluid. Proteomics. 2006;6:3445-54. PMID: 16637005 DOI: 10.1002/pmic.200500775

Liu AX, Jin F, Zhang WW, Zhou TH, Zhou CY, Yao WM, Qian YL, Huang HF. Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. Biol Reprod. 2006;75:414-20. PMID: 16738225 DOI: 10.1093/biolre/iof036

Lo Turco EG, Souza GH, Garcia JS, Ferreira CR, Eberlin MN, Bertolla RP. Effect of endometriosis on the protein expression pattern of follicular fluid from patients submitted to controlled ovarian hyperstimulation for in vitro fertilization. Hum Reprod. 2010;25:1755-66. PMID: 20427520 DOI: 10.1093/humrep/dep042

Mains LM, Christenson L, Yang B, Sparks AE, Mathur S, Van Voorhis BJ. Identification of apolipoprotein A1 in the human embryonic secretome. Fertil Steril. 2011;96:422-7.e2. PMID: 21676393 DOI: 10.1016/j.fertnstert.2011.05.049

Matorras R, Quevedo S, Corbal B, Prieto B, Exposito A, Mendoza R, Rabanal A, Díaz-Nuñez M, Ferrando M, Elortza F, Metatzaurrea A, Nagore D. Proteomic pattern of implantative human endometrial fluid in in vitro fertilization cycles. Arch Gynecol Obstet. 2018;297:1577-86. PMID: 26114977 DOI: 10.1007/s00404-018-4753-1

Missmer SA, Cramer DW. The search for biomarkers of human embryo developmental potential in IVF: a comprehensive proteomic approach. Hum Mol Reprod. 2013;19:250-63. PMID: 23247814 DOI: 10.1093/molehr/gas063

Nylawidhe J, Burch T, Bocca S, Cazares L, Green-Mitchell S, Cooke M, Birdsal P, Basu G, Semmes OJ, Oehninger S. The role of proteomics in defining the human embryonic secretome. Mol Hum Reprod. 2008;14:250-60. PMID: 18308833 DOI: 10.1093/humrep/dem426

Regiani T, Cordezo K, Carvalho VM, Perkel KJ, Zylbersztejn DS, Cedenho AP, Lo Turco EG. Follicular fluid alterations in endometriosis: label-free proteomics by MS(E) as a functional tool for endometriosis. Syst Biol Reprod Med. 2015;61:263-76. PMID: 26114977 DOI: 10.1016/j.sybrm.2015.05.003

Roth LW, McCallie B, Alvero R, Schoolcraft WB, Minijrez D, Katz-Jaffe MG. Altered microRNA and gene expression in the follicular fluid of women with polycystic ovary syndrome. Hum Reprod. 2008;90:77-83. PMID: 18281045 DOI: 10.1016/j.fertnstert.2007.11.058

Sermondade N, Dupont C, Massart P, Cédron-Durnerin I, Lévy R, Sifer C. Impact of polycystic ovary syndrome on oocyte and embryo quality. Gynecol Obstet Fertil. 2013;41:27-30. Article in French PMID: 12821065 DOI: 10.1016/j.jyobfe.2012.11.003

Silberstein T, Saphier O, Paz-Tal O, Gonzalez L, Keefe DL, Trimarchi JR. Trace element concentrations in follicular fluid represent long-term exposure. Fertil Steril. 2009;91:1771-8. PMID: 19434550 DOI: 10.1016/j.fertnstert.2008.02.007

Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancy after intracytoplasmic injection of single sperms in women: a comprehensive proteomic approach. Hum Reprod. 2013;28:1269-76. PMID: 23718701 DOI: 10.1093/humrep/deq102

Nyalawidhe J, Burch T, Bocca S, Cazares L, Green-Mitchell S, Cooke M, Birdsal P, Basu G, Semmes OJ, Oehninger S. The search for biomarkers of human embryo developmental potential in IVF: a comprehensive proteomic approach. Hum Mol Reprod. 2013;19:250-63. PMID: 23247814 DOI: 10.1093/molehr/gas063

Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnanies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340:17-8. PMID: 1351601 DOI: 10.1016/0140-6736(92)92425-F

Regiani T, Cordezo K, Carvalho VM, Perkel KJ, Zylbersztejn DS, Cedenho AP, Lo Turco EG. Follicular fluid alterations in endometriosis: label-free proteomics by MS(E) as a functional tool for endometriosis. Syst Biol Reprod Med. 2015;61:263-76. PMID: 26114977 DOI: 10.1007/s00404-018-0161-4

Salvador JM, Brown-Clay JD, Fornace AJ Jr. Gadd45 in stress signaling, cell cycle control, and apoptosis. Adv Exp Med Biol. 2013;793:1-19. PMID: 24104470 DOI: 10.1007/978-1-4614-8289-5_1

Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. Fertil Steril. 2008;90:77-83. PMID: 18281045 DOI: 10.1016/j.fertnstert.2007.11.058

Sermondade N, Dupont C, Massart P, Cédron-Durnerin I, Lévy R, Sifer C. Impact of polycystic ovary syndrome on oocyte and embryo quality. Gynecol Obstet Fertil. 2013;41:27-30. Article in French PMID: 12821065 DOI: 10.1016/j.jyobfe.2012.11.003

Silberstein T, Saphier O, Paz-Tal O, Gonzalez L, Keefe DL, Trimarchi JR. Trace element concentrations in follicular fluid of small follicles differ from those in blood serum, and may represent long-term exposure. Fertil Steril. 2009;91:1771-8. PMID: 19434550 DOI: 10.1016/j.fertnstert.2008.02.007

Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, Mardon HJ, Lokugamage AU, Rai R, Landeres C, Roelen BA, Quenby S, Kuijk EW, Kavelaars A, Heinzen CJ, Regan L, Brosens JJ, Macklon NS. Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. PLoS One. 2010;5:e10258. PMID: 20422011 DOI: 10.1371/journal.pone.010258

Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Consensus on infertility treatment related to polycystic ovary syndrome. Hum Reprod. 2008;23:462-77. PMID: 18308833 DOI: 10.1093/humrep/dem426

Thongkittidilok C, Tharasanit T, Songsasen N, Sananmuang T, Buarpung S, Techakumphu M. Epidermal growth factor improves developmental competence and embryonic quality of singly cultured domestic cat embryos. J Reprod Dev. 2015;61:269-76. PMID: 25985792 DOI: 10.1262/jrd.2014-167
Thouas GA, Dominguez F, Green MP, Vilella F, Simon C, Gardner DK. Soluble ligands and their receptors in human embryo development and implantation. Endocr Rev. 2015;36:92-130. PMID: 25548832 DOI: 10.1210/er.2014-1046

Twigt J, Steegers-Theunissen RP, Bezstarosti K, Demmers JA. Proteomic analysis of the microenvironment of developing oocytes. Proteomics. 2012;12:1463-71. PMID: 22589193 DOI: 10.1002/pmic.201100240

Wang LY, Wang DH, Zou XY, Xu CM. Mitochondrial functions on oocytes and preimplantation embryos. J Zhejiang Univ Sci B. 2009;10:483-92. PMID: 19585665 DOI: 10.1631/jzus.B0820379

Whitaker M. Calcium signalling in early embryos. Philos Trans R Soc Lond B Biol Sci. 2008;363:1401-18. PMID: 18263556 DOI: 10.1098/rstb.2008.2259

Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6:359-62. PMID: 19377485 DOI: 10.1038/nmeth.1322

Yu Y, Yan J, Li M, Yan L, Zhao Y, Lian Y, Li R, Liu P, Qiao J. Effects of combined epidermal growth factor, brain-derived neurotrophic factor and insulin-like growth factor-1 on human oocyte maturation and early fertilized and cloned embryo development. Hum Reprod. 2012;27:2146-59. PMID: 22532606 DOI: 10.1093/humrep/des099

Ziebe S, Loft A, Povlsen BB, Erb K, Agerholm I, Aasted M, Gabrielsen A, Hnida C, Zobel DP, Munding B, Bendz SH, Robertson SA. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. Fertil Steril. 2013;99:1600-9. PMID: 23380186 DOI: 10.1016/j.fertnstert.2012.12.043