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TECHNOLOGICAL INNOVATIONS

A proteomic analysis of human follicular fluid: comparison between fertilized oocytes and non-fertilized oocytes in the same patient

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Abstract
Purpose Human follicular fluid constitutes the microenvironment of follicles and includes various biological active proteins that can affect follicle growth and oocyte fertilization. Conducting proteomic evaluations of human follicular fluid may be helpful for identifying potential biomarkers possibly possessing a predictive value for oocyte quality and the success of in vitro fertilization.
Method We performed proteomic profiling of human follicular fluids containing oocytes that were fertilized and resulted in pregnancy and follicular fluids containing oocytes that were not fertilized in the same patients undergoing intracytoplasmic sperm injection using the LTQ Orbitrap coupled with liquid chromatography-tandem mass spectrometry (LC/MS/MS) analyses.
Results We identified a total of 503 proteins in human follicular fluids containing fertilized and non-fertilized oocytes obtained from 12 patients. We also found that 53 proteins exhibited significantly different spectral counts between the two groups, including heparan sulfate proteoglycan perlecan, which showed significant upregulation in the follicular fluids containing fertilized oocytes in comparison with that observed in the follicular fluids containing non-fertilized oocytes.
Conclusion Our results suggest a possibility that proteins identified by LC/MS/MS in follicular fluid might not only be involved in folliculogenesis, but also function as biomarkers possessing predictive potential for oocyte maturation and the success of IVF when their expression levels are significantly different between fertilized and non-fertilized oocytes, although no distinctive biomarkers were identified in the current study.

Keywords Follicular fluid · Heparan sulfate proteoglycan perlecan · IVF · LC/MS/MS · Proteomic analysis

Capsule A proteomic analysis using LC/MS/MS revealed significant differences in the amounts of several proteins, including heparan sulfate proteoglycan perlecan, in follicular fluid containing fertilized oocytes and that containing non-fertilized oocytes in the same patient.

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Introduction

In vitro fertilization (IVF) has been used for infertility treatment in humans since 1978. However, the success rate of IVF on the first attempt, especially in older females, is low. Predictive criteria to select the best embryo for a single embryo transfer remain elusive. Tools for selecting embryos for transfer, such as visual morphological assessments, including blastocyst and blastocoels, and measurement of the hormone concentrations in follicular fluid, are often inadequate for identifying the embryo with the best potential to establish a pregnancy [4, 14, 15, 20].

Human follicular fluid, granulosa cells and thecal cells constitute the microenvironment of follicles and influence

folliculogenesis and oocyte maturation. Several studies suggest that many human follicular fluid proteins derive from granulosa cells ([1]; Jarkovska et al.2010). Follicular fluid contains a variety of proteins that play important roles in follicle development and oocyte maturation and are thus thought to impact the outcome of in vitro fertilization. Therefore, conducting analyses of follicular fluid might be helpful in evaluating oocyte quality and identifying predictive markers for the success of IVF.

Proteomic analyses have become an important tool in the postgenomic era. Proteomics is helpful not only for identifying new disease-related biomarkers, but also for improving understanding of the pathophysiology of diseases. The application of proteomic analyses in medical research is intended to enrich a target set of disease-related proteins. In the gynecologic field, this technique has been applied in the analysis of amniotic [5, 10] and follicular fluid [3, 11, 18].

In the present study, we investigated the protein composition of human follicular fluid containing fertilized and non-fertilized oocytes obtained from 12 females undergoing in vitro fertilization using proteomic analyses with liquid chromatography-tandem mass spectrometry (LC/MS/MS).

Materials and methods

Patients

Follicular fluid (FF) samples were collected from 12 females undergoing intracytoplasmic sperm injection (ICSI) at Nagoya University Hospital. Informed consent was obtained from each patient before ovarian stimulation. This study was approved by the Ethics Committee of Nagoya University School of Medicine.

Ovarian stimulation and the IVF/ICSI procedure

Ovarian stimulation involved the administration of urinary follicle-stimulating hormone (FSH) or recombinant FSH at 150–300 IU per day for the first 2 days, after which the doses were adjusted individually based on the follicular response under gonadotropin-releasing hormone antagonists or agonists protocols. When the follicles reached 16 mm or more in mean diameter, a 10,000 IU dose of human chorionic gonadotropin (Gonadotropin; ASKA pharmaceutical, Tokyo, Japan) was administered, and oocyte retrieval was performed 35.5 h later. We classified a metaphase II (MII) oocyte with a first polar body as a mature oocyte and a metaphase I (MI) oocyte without a first polar body as an immature oocyte. The protocols used for oocyte retrieval and preparation, sperm preparation, ICSI have been described previously [25]. Next, each embryo was assessed according to the conventional criteria, as described by Veeck [27], as follows: Grade 1: blastomeres of equal size and no fragmentation, Grade 2: blastomeres of equal size with minor fragmentation, Grade 3: blastomeres of distinctly unequal size with little to no fragmentation, Grade 4: blastomeres of equal or unequal size with significant fragmentation, Grade 5: few blastomeres of any size and severe or complete fragmentation. On day 5 (114–119 h after ICSI), the embryos were scored according to the expansion of the blastocele cavity and the number and integrity of both the inner cell masses and trophectoderm cells, as previously described [13]. The patients were given 200–600 mg of a vaginal progesterone suppository daily as luteal phase support.

Preparation of follicular fluid

FF was obtained from each patient from follicles measuring 18–25 mm in diameter. We collected each oocyte and FF individually. All FF samples were individually placed into 15-mL conical tubes and centrifuged at speed (15,000 rpm) for 10 min, aliquoted into 2-mL tubes of clear supernatant and stored at −80°C until the analysis. After completion of the treated cycle, we recruited each FF containing one oocyte that was fertilized and resulted in pregnancy (fFF) and one FF containing an oocyte that was not fertilized (nfFF) in the same patients (n=12). Thereafter, we compared each of the 12 fFF to the 12 nfFF.

LC-MS/MS analysis of proteins in the FF

The 24 FF samples obtained from 12 patients were eluted using an alkylation solution by adding 10 μL of 0.1 M dithiothreitol and spraying N_2 for 1 min. The samples were left at room temperature for 30 min, then alkylated in 10 μL of 0.2 M iodoacetamide. The proteins were collected using chloroform-methanol precipitation at room temperature in the dark. The dried samples were added with 10 μL of 6 M urea and 40 μL of 0.1 M Tris-Cl, digested by a trypsin solution and incubated for 16 h at 37 °C. A nanoelectrospray tandem mass analysis was followed by LTQ Orbitrap mass spectrometry (Thermo-Fisher Scientific, San Jose, CA) combined with a paradigm MS4 HPLC system (Michrom BioResources, Auburn, CA). Aliquot samples were injected onto a reverse phase C18AQ column (0.075 mm in diameter, 50 mm in length) using the Paradigm MS4 HPLC System and eluted at a flow rate of 300 nL/min using 2 % acetonitrile with 0.1 % formic acid and 90 % acetonitrile with 0.1 % formic acid. The mass spectrometer was equipped with an XYZ interface (AMR, Tokyo, Japan), and a precursor ion scan was carried out using a 400–1,500 mass-to-charge (m/z) ratio prior to the MS/MS analysis.

The samples were searched using the NCBI human protein reference database and analyzed using the Mascot software program (Matrix Science, London, UK; version mascot). The MS/MS data were validated using a Scaffold data analysis (version Scaffold_3_00_03, proteome Software Inc, Portland, OR). The total number of spectra for each protein per condition was generated using the 'normalized number of unique spectra' option. Scaffold uses X! Tandem, Protein Prophet and Peptide

Prophet [22] to sequence the MS/MS data. The Scaffold data analysis consisted of a quantitative comparison followed by a 95 % peptide identification probability analysis and a 99 % protein identification probability analysis that contained two or more identified peptides [7]. Proteins that contained similar peptides and could not be differentiated based on an MS/MS analysis alone were grouped together to satisfy the principles of parsimony.

Statistical analysis

All statistical analyses were performed using the SPSS analysis software program (Dr. SPSS II LEADTOOLS 1991–2000. LEAD Technologies. Inc., USA). We used the Mann–Whitney *U*-test to identify the number of unique spectra in the fertilized and non-fertilized oocyte follicular fluid groups. *P*-values less than 0.05 were considered to be significant.

Results

Patient characteristics and identification of proteins in human follicular fluid using LTQ-Orbitrap MS/MS

We described the patient characteristics and cycle outcomes in Table 1. The mean patient age (mean±SD) was 34.8± 4.6 years.

Using the LTQ-Orbitrap, hundreds of proteins were identified in each sample. The number of proteins identified by the LTQ-Orbitrap in 0.1 mg of each sample was used in a database search with MASCOT and X! Tandem, as listed in Table 2. We did not find any significant differences in the numbers of identified proteins in FF between the fertilized and non-fertilized groups.

Scaffold data analysis and a summary of identified proteins, peptides and spectra in the fertilized and non-fertilized groups

A total of 503 proteins were identified in 24 human follicular fluid samples using a Scaffold data analysis in the present study (Supplemental Table 1). The albumin and immunoglobulin families together represented over 80 % of the total proteins in the FF samples. A bioinformatics analysis was performed on the FF proteome. A biological process analysis demonstrated that 444 proteins belonged to the unknown function group. The other proteins were classified as follows: two proteins in the developmental process group, four proteins in the signal transduction group, nine proteins in the localization group and 52 proteins in the metabolic process group. The MS/MS spectra were searched via the NCBI human database using the MASCOT software

Table 1 Patient characteristics

	Age (year)	BMI (kg/m^2)	Prior attempted cycles (*n*)	Infertility diagnosis	No. of oocytes collected (*n*)	Sperm count (total, × 10^6/ml)	Sperm count (motile, × 10^6/ml)	No. of oocytes fertilized (*n*)	Oocyte stage (fertilized/non-fertilized)	Day of embryo transfer	Grade of embryo	Outcome	Ovarian stimulation protocol
1	37	20.3	2	unexplained	12	140.6	69.3	6	MII/D	day 6	BC	LB	ant
2	35	18.8	9	PCOS	12	43.3	19.6	4	MII/D	day 3	G4	AB	ago
3	32	21.7	0	unexplained	8	47.0	22.0	4	MII/MII	day 6	CC	LB	ago
4	45	19.2	4	tubal	6	57.3	23.6	6	MII/MII	day 2	G1	AB	ant
5	39	28.3	3	PCOS	6	92.3	43.6	4	MII/MII	day 2	G1	LB	ago
6	30	18.8	3	PCOS	27	37.0	13.3	6	MII/MI	day 2	G1	LB	ago
7	37	23.5	1	tubal	6	48.6	26.0	3	MII/MI	day 2	G1	AB	ant
8	29	15.4	0	PCOS	28	96.0	42.0	6	MII/D	day 3	G1	AB	ago
9	33	23.8	1	male	11	0.2	0.1	8	MII/MI	day 5	AC	LB	ant
10	38	22.1	0	tubal	8	60.0	36.0	2	MII/MII	day 3	G1	LB	ago
11	32	20.0	0	unexplained	13	126.6	77.3	10	MII/MI	day 3	G2	LB	ago
12	30	22.5	2	unexplained	11	73.3	58.3	5	MII/MI	day 2	G2	AB	ant

BMI body mass index; *PCOS* polycystic ovary syndrome; *MII* metaphase II; *D* degeneration; *MI* metaphase I; *LB* live birth; *AB* abortion; *ant* antagonist; *ago* agonist

Table 2 Numbers of proteins in human follicular fluid identified using LTQ-Orbitrap

Patient	Proteins identified (Mascot)	
	fFF	nfFF
1	935	933
2	1,129	816
3	1,156	1,149
4	1,024	1,140
5	1,646	1,597
6	1,049	1,335
7	799	784
8	657	609
9	1,541	1,089
10	1,343	1,017
11	936	851
12	1,131	553

fFF follicular fluid containing fertilized oocytes; *nfFF* follicular fluid containing non-fertilized oocytes

program, and the data were compiled in a Scaffold file for viewing and the statistical analysis. We compared the identified proteins, peptides and spectra between the two groups using a Scaffold analysis (Fig. 1). A complete list of the proteins identified in both the fertilized and non-fertilized FF samples was compiled via Scaffold, and a quantitative comparison was made followed by a 95 % peptide identification probability analysis and a 99 % protein identification probability analysis that contained two or more identified peptides. Four-hundred and seventy-six proteins were identified in both groups, 18 proteins were identified in the fertilized group only and nine proteins were identified in the non-fertilized group only (Fig. 1a). Venn diagrams of the peptides and spectra are shown in Fig. 1b and c. The lists of the proteins identified in the fertilized or non-fertilized groups only are shown in Tables 3 and 4.

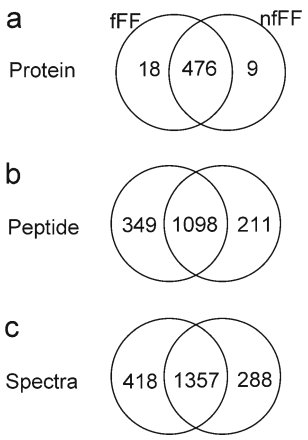

Fig. 1 Venn diagrams depicting the overlap of proteins (**a**), peptides (**b**) and spectra (**c**) differentially expressed in the follicular fluids containing fertilized oocytes (*fFF*) and non-fertilized oocytes (*nfFF*)

The Scaffold program quantified the spectral counts of each protein. We analyzed the spectral counts of the 476 proteins identified in both groups using the SPSS software program. Fifty-three proteins were found to be significantly different in spectral count between the two groups (Table 5).

Heparin sulfate proteoglycan perlecan was upregulated in the fertilized oocyte group

HSPG is ubiquitously distributed on the surface of animal cells and exhibits many important biological activities such as growth factor binding and apoptosis regulation [8]. In this study, we found that the expression of HSPG was significantly different between the two groups: a higher expression level was observed in the fertilized-oocyte follicular fluid group based on the spectral count (Fig. 2).

Table 3 Differentially expressed proteins in the human fertilized follicular fluid identified by LC/MS/MS

Protein name	NCBI accession number	Function
Fibronectin-binding protein A	FNBA_STAA8	Cell adhesion
Protein yibA	YIBA_ECO57	Unknown
Acetate kinase 1	ACKA_ANAVT	Kinase Transferase
lens gamma-s-crystallin	gi|1362852	Unknown
Mitochondrial enolase superfamily member 1	ENOF1_XENLA	Isomerase activity Metal ion binding
Phosphoprotein phosphatase	gi|227878881	Cell cycle
5-methylcytosine-specific restriction endonuclease	gi|300310547	Hydrolase
hCG2040526	gi|119587144	Unknown
Ribosomal RNA small subunit methyltransferase G	RSMG_SHEAM	Methyltransferase Transferase
Immunoglobulin heavy chain variable region	gi|118406175	Immune response
Agmatine deiminase	AGUA_PSEFS	Polyamine biosynthesis
Immunoglobulin heavy chain variable region	gi|14589225	Immune response
Immunoglobulin kappa chain variable region	gi|14268408	Immune response
Immunoglobulin kappa light chain variable	gi|1568567	Immune response
Immunoglobulin kappa chain	gi|186048	Immune response
Immunoglobulin kappa light chain variable region	gi|98956324	Immune response
Chain A	gi|171848737	Unknown
tripartite motif-containing 43	gi|119591726	Intracellular Zinc ion binding

Table 4 Differentially expressed proteins in the human non-fertilized follicular fluid identified by LC/MS/MS

Protein name	NCBI accession number	Function
immunoglobulin kappa light chain variable region	gi|70797833	Immune response
conserved hypothetical protein	gi|304437553	Complete proteome
putative transcriptional regulator	gi|125623972	Transcriptional regulator
diaminopropionate ammonia-lyase	gi|157162332	Pyridoxal phosphate binding
50S ribosomal protein L14	gi|57238704	Translation
GTP-binding protein	YPT53_YEAST	Protein transport
anti-HPA-5b	gi|37780335	Immune response
unnamed protein product	gi|34531444	Unknown
selenoprotein T precursor	gi|42789380	Unknown

Discussion

Some substances in human follicular fluid that are involved in folliculogenesis are produced by granulosa cells, thecal cells and oocytes. Therefore, exploring the components in follicular fluid may contribute to identifying biomarkers of follicular maturation, which might lead to the ability to predict the probability of becoming pregnant. The oocytes

Table 5 The list of identified significant differentially expressed proteins between fertilized follicular fluid and non-fertilized follicular fluid identified by LC/MS/MS

Protein name	NCBI accession number	*P*	Function
Alpha-2-antiplasmin	gi|115583663	0.03	Protease inhibitor Acute-phase response Blood vessel morphogenesis Collagen fibril organization
Anti-pneumococcal capsular polysaccharide	gi|15637417	0.04	Unknown
Anti-tetanus toxoid	gi|58222664	0.03	Unknown
Apolipoprotein A-IV	gi|71773110	0.04	Cholesterol metabolic process Phosphatidylcholine metabolic process
			Regulation of cholesterol transport
			Leukocyte adhesion
Cellulose synthase-like protein H2	CSLH2_ORYSJ	0.04	Cellular cell wall organization Cellular biosynthetic process
Chain A	gi|157831597	0.04	Unknown
Chain B	gi|298508666	0.04	Unknown
Chain C	gi|166007160	0.02	Unknown
Chain H	gi|15825647	0.04	Unknown
Chain H	gi|223673814	0.04	Unknown
Complement component 9	gi|119576390	0.03	Complement pathway Complement alternate pathway
Fibrin beta	gi|223002	0.02	Cell cortex platelet activation Signal transduction Response to calcium ion Fibrinogen complex
Immunoglobulin heavy chain variable region	gi|66821275	0.04	Immunoglobulin domain
hCG2001591	gi|119623974	0.04	Unknown
Heparan sulfate proteoglycan perlecan	gi|11602963	0.04	Cell adhesion
			Angiogenesis
			Brain development
			Cardiac muscle tissue development protein localization
Hypothetical protein	gi|11602963	0.03	Unknown
Ig G1 H Nie	gi|229601	0.03	Unknown
Ig heavy chain variable region, VH3 family	gi|33319266	0.04	Unknown
Ig kappa chain V region	gi|480931	0.04	Immune response
Immunoglobulin heavy chain	gi|29836821	0.02	Immune response
Immunoglobulin heavy chain	gi|219566071	0.04	Immune response

Table 5 (continued)

Protein name	NCBI accession number	*P*	Function
Immunoglobulin heavy chain variable region	gi|4107050	0.04	Immune response
Immunoglobulin heavy chain variable region	gi|247425015	0.03	Immune response
Immunoglobulin heavy chain variable region	gi|19744498	0.03	Immune response
Immunoglobulin heavy chain variable region	gi|247424935	0.02	Immune response
Immunoglobulin heavy chain variable region	gi|19744542	0.04	Immune response
Immunoglobulin kappa chain	gi|722432	0.04	Immune response
Immunoglobulin kappa chain variable region	gi|77379444	0.02	Immune response
Immunoglobulin kappa chain variable region	gi|5578800	0.03	Immune response
Immunoglobulin kappa heavy chain	gi|2765421	0.04	Immune response
Immunoglobulin kappa light chain V region	gi|2599536	0.04	Immune response
Immunoglobulin kappa light chain variable region	gi|4323984	0.02	Immune response
Immunoglobulin lambda 6 light chain	gi|170684384	0.02	Immunoglobulin domain
Inter-alpha inhibitor H2	gi|119606782	0.02	Protease inhibitor Hyaluronan metabolic process
Inter-alpha inhibitor H4	gi|119585669	0.02	Acute-phase response Hyaluronan metabolic process
Plasminogen	gi|38051823	0.02	Fibrinolysis Proteolysis Regulation of cell proliferation Blood coagulation
Complement C4-B	gi|81175167	0.02	Complement pathway Inflammatory response
Ig kappa chain V-I	gi|125761	0.03	Immunoglobulin domain Complement activation Classical pathway
R-spondin-3	RSPO3_HUMAN	0.04	Sensory transduction Heparin binding
Ubiquinone biosynthesis monooxygenase COQ6	COQ6_HUMAN	0.04	Golgi apparatus Cell projection Mitochondrion
Unnamed protein product	gi|34536000	0.01	Unknown
Unnamed protein product	gi|16554039	0.01	Unknown
Unnamed protein product	gi|14042015	0.01	Unknown
Unnamed protein product	gi|21758945	0.04	Unknown
Unnamed protein product	gi|21757089	0.01	Unknown
Unnamed protein product	gi|194376234	0.04	Unknown
Unnamed protein product	gi|158259769	0.04	Unknown
Unnamed protein product	gi|34532520	0.002	Unknown
Unnamed protein product	gi|34527290	0.04	Unknown
Unnamed protein product	gi|34535625	0.04	Unknown
Unnamed protein product	gi|221039462	0.01	Unknown
Unnamed protein product	gi|34535045	0.03	Unknown
Vitronectin	gi|13477169	0.01	Cell adhesion Immune response Negative regulation of blood coagulation

present in the milieu of FF, which is rich in hormones such as progesterone, FSH and luteinizing hormone (LH), are associated with follicular maturation and oocyte development [17]. Epidermal growth factor (EGF) [2], insulin-like growth factor (IGF) and transforming growth factor (TGF) [6] have been detected in follicular fluid.

There have been several reports of proteomic analyses using human follicular fluids. Hanrieder et al. identified 69 proteins in the follicular fluids of seven females obtained during IVF using isoelectric focusing and reversed-phase nanoliquid chromatography coupled to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry [16]. Jarkovska et al.

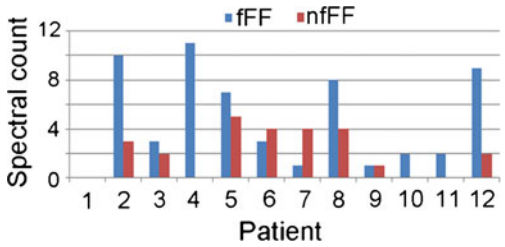

Fig. 2 Spectral counts of heparan sulfate proteoglycan perlecan in the follicular fluids containing fertilized oocytes (*fFF*) and non-fertilized oocytes (*nfFF*) in each patient (1–12)

identified differentially expressed proteins between follicular fluids and plasma obtained from females undergoing successful IVF using 2-D electrophoresis and 2-D liquid chromatography [18]. Twigt et al. identified 246 specific proteins in two samples of follicular fluid using LC/MS/MS [26]. Estes et al. performed a proteomic evaluation of follicular fluids to compare good responders and poor responders in matched pairs of IVF patients [11]. Spitzer et al. found no major differences in the protein patterns of various mature follicles using up to 60 protein spots identified with two-dimensional electrophoresis [24].

In this study, we investigated human fertilized and non-fertilized follicular fluid obtained from females undergoing IVF using the LC/MS/MS technique in 12 pairs of patient samples. To the best of our knowledge, this is the first report comparing one FF containing an oocyte that was fertilized and resulted in pregnancy and FF containing an oocyte that was not fertilized in the same patient. The number of proteins indentified in the current study was higher than that reported in a previous study of a proteomic analysis using follicular fluids. We quantified and compared 503 proteins identified in human fertilized and non-fertilized follicular fluid obtained from 12 patients. Differences in the proteins expressed in fertilized and non-fertilized FF were assessed: 18 proteins were found in fertilized FF and nine proteins were found in non-fertilized FF. However, these proteins were not always expressed in all 12 cases. The proteins found only in follicular fluid containing either fertilized oocytes or non-fertilized oocytes showed low levels of expression, which might indicate a lack of significance as a biomarker of oocyte quality. Therefore, we investigated the expression levels of the proteins identified in both groups.

In LC/MS/MS analyses, the spectral counts of proteins significantly vary with respect to the expression level of each protein [7]. Spectral counting was applied in this study for high-throughput quantification of the total number of spectra. We compared 476 proteins whose total numbers of spectra were identified in both groups using an SPSS statistical analysis. A total of 53 proteins were found to be significantly different between the two groups in our study. Vitronectin is a multifunctional adhesive glycoprotein that plays a significant role in a number of physiological processes such as cell adhesion, cell migration, cell invasion, humoral defense mechanisms, modulation of the immune system and regulation of the plasminogen activation system [23]. Apolipoprotein A-IV, a glycoprotein, is a member of the apoA1/C3/A4/A5 gene cluster that is synthesized by the small intestine and involved in lipid and lipoprotein metabolism [9]. Although the precise function of apolipoprotein A-IV is not known, several studies have suggested that it is associated with cardiovascular disease and might be involved in reverse cholesterol transport, which is important for cholesterol homeostasis and steroidogenesis [21]. The apolipoprotein A-IV present in human follicular fluid might be implicated in the regulation of lipid homeostasis and steroidogenesis during oocyte maturation.

We identified HSPG in human follicular fluid. Furthermore, HSPG was found to be expressed at a significantly high level in the fertilized FF group in an LC/MS/MS analysis using spectral counting in our study. HSPG can bind bone morphogenetic protein-2 (BMP-2) of the transforming growth factor-β (TGF-β) superfamily and thereby mediate BMP-2 internalization in osteoblasts, which results in the regulation of BMP-2 osteogenic activity [19]. As for folliculogenesis, members of the TGF-β superfamily, such as growth differentiation factor-9 (GDF-9), have been reported to be significantly implicated in this process. In addition, types of HSPG, including syndecans and glypicans, are expressed in ovarian granulosa cells and released under gonadotropin regulation [8]. In periovulatory follicles, HSPG possibly acts as a coreceptor mediating oocyte secreted factors and therefore plays an essential role in GDF9 signaling and is involved in the patterning of oocyte signaling and the cumulus cell function [28]. Perlecan is a core protein of HSPG that supports multiple biological activities that are relevant to embryonic development such as cell adhesion, growth factor binding and apoptosis regulation [12]. The high level of HSPG perlecan expression in fertilized FF observed in this study might impact oocyte fertilization.

In this report, we present the findings of a proteomic analysis of human fertilized and non-fertilized follicular fluid in females undergoing ICSI using an LC/MS/MS analysis. Our study suggests that several proteins found in human follicular fluid, including HSPG, might be used as protein biomarkers of oocyte quality, which is possibly implicated in the prediction of fertilization, although we failed to identify the distinctive biomarkers. In the current study, we first demonstrated that there are significant differences in the amounts of several proteins identified in LC/MS/MS between FF containing an oocyte that was fertilized and resulted in pregnancy and FF containing an oocyte that was not fertilized in the same patient. Furthermore, the number of proteins indentified in the current study was higher than that reported in a previous study of a proteomic analysis using follicular fluids. However, a limitation of this study is that we are unable to attribute fertilization to oocyte quality only, although we

tried to minimize the influence of sperm quality using ICSI. An increased number of samples would enable the use of a proteomics analysis to identify distinctive biomarkers. In addition, follicular fluids comprise the microenvironment of follicles; therefore, a proteomics analysis may reveal differences in follicular fluids regarding polycystic ovary syndrome and poor responses. All things considered, further study is required to extend and strengthen our findings.

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Conflict of interest The authors declare that they have no conflict of interest.

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