Available online at www.tox.or.kr

Identification of Proteins in Human Follicular Fluid by Proteomic Profiling

Young-Jin Sim[1,2] **& Mi-Young Lee**[2]

[1]Mirae & Woman OBGY's Hospital, Susong-dong, Gunsan,Jeollabuk-do 573-370, Korea
[2]Department of Medical Biotechnology, SoonChunHyang University, Asan, Chungnam 336-600, Korea
Correspondence and requests for materials should be addressed to M. Y. Lee (miyoung@sch.ac.kr)

Accepted 13 June 2008

Abstract

Human follicular fluid (HFF) is the *in vivo* microenvironment for oocyte maturation and includes a variety of proteins that could be involved in oocyte development and fertilization. We therefore used a proteomic approach to identify new HFF proteins. HFF from mature human follicles was obtained from five women following oocyte collection for in vitro fertilization (IVF). Ethanol-precipitated HFF run on two-dimensional gel electrophoresis (2DE) produced approximately 250 Coomassie brilliant blue-stained spots, 64 of which were identified using matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). In this study, several proteins including complement factor H, inter-α (globulin) inhibitor H4, inter-α-trypsin inhibitor heavy chain H4 precursor, human zinc-α-2-glycoprotein chain B, PRO2619, PRO02044, and complex-forming glycoprotein HC were new proteins that have not been previously reported in HFF using proteomic methods. Additionally, we identified alloalbumin venezia for the first time from trichloroacetic acid (TCA)-precipitated HFF. These HFF proteins could serve as new biomarkers for important human reproductive processes.

Keywords: Human follicular fluid, Proteomics, Two-dimensional gel electrophoresis, MALDI-MS

Human follicular fluid (HFF) contains a wide variety of biologically active molecules such as carbohydrates, mucopolysaccharides, lipids, steroids, peptide hormones, and growth factors. The chemical composition of HFF may reflect oocyte developmental stages, and specific components of HFF could be used as indicators of various secretory or metabolic activities of follicular cells[1]. In the human reproductive system, HFF has been suggested to play an essential role in follicular maturation and oocyte fertilization[2]. Some HFF proteins produced by granulosa and thecal cells in the ovary have been suggested to regulate oocyte maturation during follicular development[3]. Therefore, HFF has been used as a source for identifying proteins involved in human folliculogenesis[3].

Due to the molecular complexity of HFF, which is not unlike blood plasma, sensitive and high-resolution techniques are necessary to analyze HFF proteins. Previous proteomic approaches to identify HFF proteins have detected a number of proteins. Recently, Hanrieder *et al*. identified sex hormone binding globulin and inhibin A in HFF by using nano-liquid chromatography (LC) matrix-assisted laser desorption /ionization time-of-flight (MALDI TOF)/TOF-mass spectrometry (MS)[4]. Transferrin, ceruloplasmin, afamin, hemopexin, haptoglobulin, and amyloid protein have also been identified in HFF using two-dimensional gel electrophoresis (2DE)[5]. Transcripts for hormone sensitive lipase, unnamed protein product 1, unnamed protein product 2, and apolipoprotein A-IV precursor were also discovered in HFF using reverse transription-PCR, and these genes were shown to be derived from human primary granulosa cells[6]. Complement component C3c chain E, fibrinogen gamma, antithrombin, angiotensinogen, and hemopexin precursor were identified with MALDI-TOF-MS and nano-LC MS/MS and were aberrantly expressed in HFF from recurrent spontaneous abortion patients[7]. Thioredoxin peroxidase 1, transthyretin, and retinol-binding protein were also identified and suggested to be potential candidates for specific functions during folliculogenesis[8]. Despite this progress, only a very small portion of the entire HFF proteome has been revealed, and the vast majority is still unknown. Therefore, we hypothesized the need to discover new HFF proteins that might provide a better understanding of human reproductive processes and pathologies during fertilization and oocyte development.

To increase our knowledge of the protein components of follicular fluids, we analyzed total proteins

in HFF via proteomics. When we compared our data to other 2-DE protein profiles published for HFF, we isolated some proteins that have not been reported previously.

HFF contains serum-derived proteins as well as proteins from granulosa and thecal cells. Granulosa cells take up follicular fluid into small cavities that fuse into a single cavity, thus accumulating follicular fluid from all phases of follicular growth[8]. Because the blood-follicular barrier becomes more permeable to plasma proteins during maturation, proteins common to both compartments can be found in HFF. It has been suggested that some HFF proteins play an important role in the regulation of follicular maturation[9,10], and some of these proteins could be used as biomarkers for oocyte maturation during follicular development[3,11]. The protein expression patterns for mature HFF from different individuals are very similar, whereas considerable differences exist between mature and immature HFF[12]. Previously, by comparing the intensity of Coomassie blue-stained spots from different individuals, we found essentially conserved HFF protein expression patterns with only a few minor differences (data not shown). In the present study, HFF proteins were precipitated with ethanol or TCA, separated on 2-DE gels, and identified using MALDI-MS.

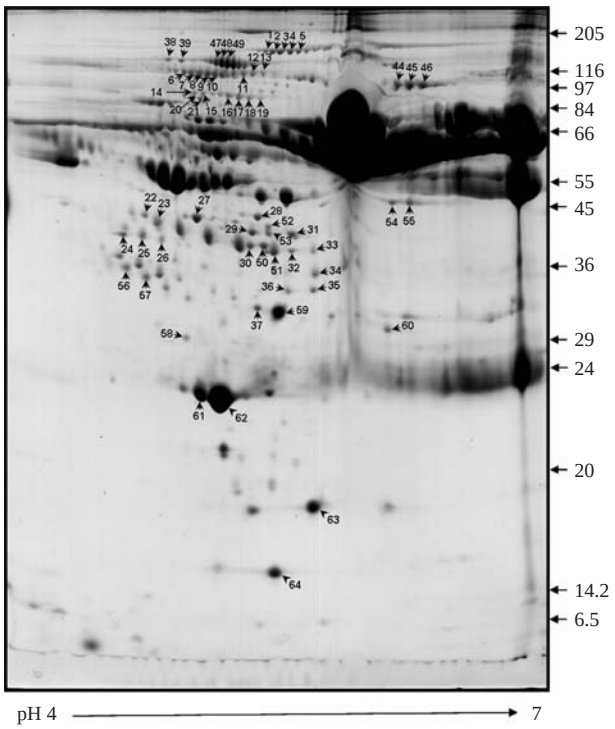

Figure 1. 2-dimensional gel electrophoregram of the proteins in human follicularfluid following ethanol precipitation.

Proteomic Profiling of Human Follicular Fluid

A representative 2-DE map of Coomassie brilliant blue-stained HFF proteins having isoelectric points (pIs) in the 4-7 pH range and molecular weights (MWs) between 10 and 205 kDa shows that most HFF proteins have pIs between pH 4 and 7 (Figure 1). In total, approximately 250 HFF protein spots were resolvable using computer-aided image analysis of the 2-DE gel. We positively identified the proteins in 64 of these spots (Table 1). Overall protein expression profiles of three replicates of each sample were almost identical, indicative of high reproducibility.

We identified afamin (spots 20, 21), clusterin (spots 56, 57), α1-antitrypsin (spots 38, 39), haptoglobin (spots 23, 33), apolipoprotein E (spot 37), transthyretin (spot 59, 64), proapolipoprotein (spots 61, 62), haptoglobin α2 chain (spot 63), and apolipoprotein A-IV precursor (spot 27), all with similar pIs and MWs as previously reported[4-8].

In addition, we identified eight new proteins by 2-DE/MALDI-TOF MS that have not previously been reported in mature HFF: complement factor H (spots 1, 2, 3, 4, 5), inter-α (globulin) inhibitor H4 (spots 6, 10, 11, 13), inter-α-trypsin inhibitor heavy chain H4 precursor (spots 7, 8, 9, 12), human zinc-α-2-glycoprotein chain B (spots 25, 26), PRO2619 (spots 28, 55), PRO02044 (spots 34, 35, 36), and complex-forming glycoprotein HC (spot 58). Moreover, we separated and identified alloalbumin venezia (spots 40, 41, 42, 43) as a new HFF protein by using 10% TCA rather than 80% ethanol for protein precipitation (Figure 2). In the 80% ethanol precipitates, the proteins running near alloalbumin venezia were not well resolved on 2-DE gels, but the separation of the spots in this region of the gel was much improved in the 10% TCA precipitates (Figure 2, upper left region). We suspect that they missed identifying complement factor H, inter-α (globulin) inhibitor H4, and inter-α-trypsin inhibitor heavy chain H4 precursor in previous studies[6-8] because they have MWs >100 kDa, too large to be resolved on their previous 2-DE gels.

Discussion

Spots 1 to 5 contained complement factor H, which plays an essential role in the homeostasis of the complement system and the protection of tissues from damage during complement activation[13]. Several recent studies have described an association of complement factor H gene (CFH) genetic variations with aty-

Table 1. Proteomic identification of the protein in human follicular fluid.

Spot No.	Identified protein	Accession No.	Cov.%	Matching peptide No.	M.W/pI
1	Complement factor H	NP_000177	22	25	143,790/6.3
2	Complement factor H	NP_000177	25	29	143,790/6.3
3	Complement H factor	AAA52016	24	15	76,680/6.4
4	Complement factor H	NP_000177	24	22	143,790/6.3
5	Complement factor H	NP_000177	18	21	143,790/6.3
6	Inter-alpha (globulin) inhibitor H4	NP_002209	12	11	103,570/6.5
7	Inter-alpha-trypsin inhibitor heavy chain H4 precursor	Q14624	23	18	103,570/6.5
8	Inter-alpha-trypsin inhibitor heavy chain H4 precursor	Q14624	26	23	103,570/6.5
9	Inter-alpha-trypsin inhibitor heavy chain-related protein precursor	1082547	28	25	103,600/6.5
10	Inter-alpha (globulin) inhibitor H4	NP_002209	20	16	103,570/6.5
11	Inter-alpha (globulin) inhibitor H4	NP_002209	28	23	103,570/6.5
12	Inter-alpha-trypsin inhibitor heavy chain-related protein precursor	JX0368	27	23	103,600/6.5
13	Inter-alpha (globulin) inhibitor H4	NP_002209	24	18	103,570/6.52
14	Complement C4A precursor	2144577	13	22	194,440/6.7
15	Complement C4 precursor	P01028	18	25	194,350/6.7
16	Complement C4 precursor	P01028	14	20	194,350/6.7
17	Complement C4 precursor	P01028	12	19	194,350/6.7
18	Complement C4 precursor	P01028	17	24	194,350/6.7
19	Complement C4A precursor	2144577	13	18	194,440/6.7
20	Afamin precursor	NP_001124	28	16	70,990/5.6
21	Afamin precursor	NP_001124	30	17	70,990/5.6
22	Serum paraoxonase	AAA60142	32	9	37,990/5.0
23	Haptoglobin	AAC27432	26	9	38,730/6.1
24	Complement component 3 precursor	NP_000055	6	11	188,690/6.0
25	Chain B, Human Zinc-α2-Glycoprotein	1ZAGB	40	13	31,860/5.7
26	Chain B, Human Zinc-α2-Glycoprotein	1ZAGB	32	10	31,860/5.7
27	Apolipoprotein A-IV precursor	AAA51748	57	27	47,370/5.2
28	PRO2619	AAG35503	13	7	58,530/6.0
29	Similar to human albumin	AAA64922	24	13	53,430/5.7
30	ALB protein	AAH41789	47	19	48,650/6.0
32	ALB protein	AAH41789	33	12	48,650/6.0
33	Haptoglobin	AAC27432	26	8	38,730/6.1
34	PRO02044	AAF22034	38	8	30,090/7.0
35	PRO02044	AAF22034	40	11	30,090/7.0
36	PRO02044	AAF22034	53	14	30,090/7.0
37	Apolipoprotein E precursor	NP_000032	50	16	36,250/5.6
38	Chain A, A 2.1 Angstro m structure of an uncleaved α-1-antitrypsin shows variability of the reactive center and other loops	1HP7A	25	10	44,320/5.4
39	Alpha-1-antitrypsin precursor	P01009	46	17	46,890/5.4
40	Alloalbumin venezia	AAA98798	19	13	71,210/6.0
41	Chain A, crystal structure of human serum albumin	1AO6A	38	22	68,450/5.7
42	Alloalbumin venezia	AAA98798	36	20	71,210/6.0
43	Alloalbumin venezia	AAA98798	40	23	71,210/6.0
44	Complement factor B, preproprotein	AAH04143	27	17	86,910/6.6
45	Complement factor B, preproprotein	AAH04143	33	22	86,910/6.6
46	Complement factor B preproprotein	NP_001701	40	28	86,860/6.6
47	X-ray crystal structure of human ceruloplasmin at 3.0 angstroms	1KCW	15	18	120,870/5.4
48	X-ray crystal structure of human ceruloplasmin at 3.0 angstroms	1KCW	14	17	120,870/5.4
49	X-ray crystal structure of human ceruloplasmin at 3.0 angstroms	IKCW	24	19	120,870/5.4
50	ALB protein	AAH41789	38	14	48,650/6.0
51	Haptoglobin precursor, allele 1	67586	27	11	38,950/6.1
52	Chain A, crystal structure of human serum albumin	1AO6A	24	14	68,450/5.7
53	Chain A, crystal structure of human serum albumin	1AO6A	22	13	68,450/5.7
54	Serum albumin	CAA23754	30	15	71,340/6.1

Table 1. Continued.

Spot No.	Identified protein	Accession No.	Cov.%	Matching peptide No.	M.W/pI
55	PRO2619	AAG35503	34	14	58,530/6.0
56	Clusterin	AAP88927	15	7	52,790/6.1
57	Clusterin	AAP88927	15	6	52,790/6.1
58	Complex-forming glycoprotein HC	0801163A	35	5	20,590/5.8
59	Chain 1, The X-ray crystal structure refinements of normal human transthyretin and the amyloidogenic val 30	1ETA1	77	7	13,930/5.3
60	Chain A, human serum albumin mutant R218p complexed with thyroxine	1HK3A	18	11	68,390/5.6
61	Proapolipoprotein	AAA51747	48	15	28,940/5.4
62	Proapolipoprotein	AAA51747	67	23	28,940/5.4
63	Haptoglobin Hp2	1006264A	22	5	42,350/6.2
64	Chain A, an engineered transthyretin monomer that is non-Amyloidogenic-unless partially denatured	1GKOA	61	5	13,800/5.3

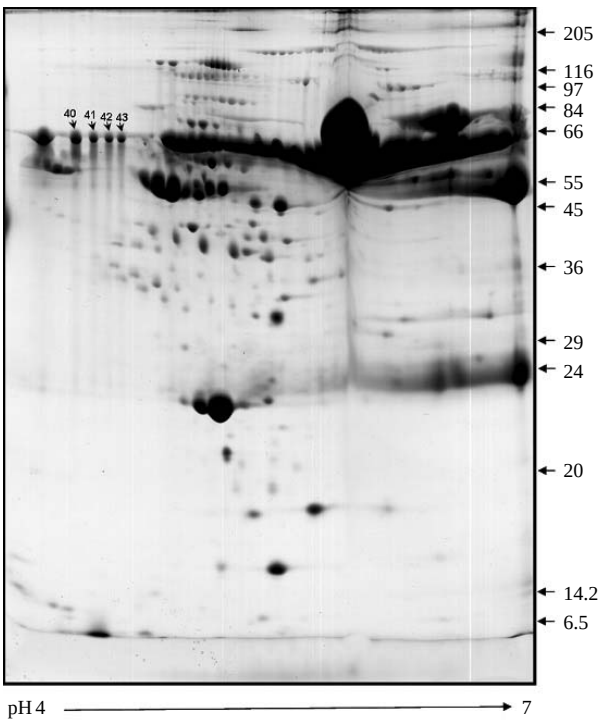

Figure 2. 2-dimensional gel electrophoregram of the proteins in human follicular fluid following trichloroacetic acid precipitation.

pical hemolytic uremic syndrome (aHUS), age-related macular degeneration (AMD), and membranoproliferative glomerulonephritis (MPGN)[14]. CFH seems to play a role in inflammation and may therefore protect cells during oocyte maturation.

Inter-α (globulin) inhibitor, also known as the inter -α-trypsin inhibitors (ITIs), a family of plasma protease inhibitors assembled from a light chain bikunin and five homologous heavy chains (ITI, H1-5)[15], exhibited some variability between spots.

Spots 6, 10, 11 and 13 were determined to be ITI H4, and spots 7, 8, 9 and 12 were determined to be ITI H4 precursor. ITIs also play an important role in inflammation as well as extracellular matrix stability and carcinogenesis. ITI inhibits complement activation, complement-dependent phagocytosis, and complement-dependent lung injury[16]. Moreover, ITI has been classified as an osmotically active molecule in follicular fluid, so it could be involved in regulating the follicular fluid osmotic gradient across the follicular wall[17]. ITI binds to hyaluronan in a complex with tumor necrosis factor stimulated gene-6 during the expansion process of ovulation[17,18]. Thus, ITI appears to be an essential component of the matrix for the release of the cumulus oocyte complex through the ovulation pore[18].

Spot 58 was complex-forming glycoprotein HC (heterogeneous in charge). This glycoprotein has been found in human plasma, urine, and cerebrospinal fluid[19], but this is the first report showing its presence in HFF. The protein is closely related to α1-microglobulin, and their sequences differ by only a single amino acid[20]. Moreover, because the protein occurs as a free monomer, dimers and in complexes with IgA and albumin, complex-forming glycoprotein HC has already been suggested as a biomarker for immunoregulation[21].

Spots 25 and 26 contained human zinc-α-2-glycoprotein (ZAG) chain B. ZAG is an inflammatory biomarker linking between obesity and associated pathologies[22]. This protein stimulates lipid degradation in adipocytes and causes extensive fat loss under both normal or pathological conditions. Recently, the protein activity was found to be mediated via adrenore-

ceptors with upregulation of the cAMP/cAMP-dependent protein kinase A signaling pathway. Moreover, the possibility that ZAG may play a role in the regulation of sperm motility has also been suggested[23].

We discovered various albumin domains including alloalbumin venezia (spots 40, 41, 42, 43), PRO2619 (spots 28, 55), PRO02044 (spots 34, 35, 36), human serum albumin chain A (spots 52, 53), albumin analog (spot 29), and ALB protein (spots 30, 32, 50). The detection of these albumin fragments in the HFF does not appear to be an analytical artifact, because a variety of body fluids such as cerebrospinal fluid, amniotic fluid, and bronchoalveolar lavage fluid also contain albumin fragments[24,25].

The complete identification of the HFF proteome is of major importance and has potential clinical implications. Our results provide a standard 2-DE map of the normal mature HFF proteins, establish the presence of several new HFF proteins, and may contribute to the identification of reliable biomarkers for oocyte competence. Moreover, it would be useful to obtain marker proteins in HFF to effectively study some of the physiological changes during oocyte maturation and to diagnose ovary-related diseases.

Materials and Methods

Human Follicular Fluid Preparation

Human follicular fluids (HFFs) were obtained from five women (average age of 35 yr) undergoing in vitro fertilization at MizMom OBGY's Hospital (Cheonan City, Korea) and Mirae & Woman OBGY's Hospital (Gunsan City, Korea). When the diameters of at least two follicles were greater than 18 mm during the controlled ovarian hyperstimulation, 10,000 IU of human chorionic gonadotropin (hCG; LG Chemical Co. Korea) were administered. Oocyte retrieval was performed 35 hr later through transvaginal ultrasound-guided follicle aspiration. HFF was collected and centrifuged at 13,000 rpm for 30 min to remove granulosa cells and blood from the follicular fluid. The supernatants were collected and only microscopically clear, blood-free yellow follicular fluids were used for this study. Informed consent was obtained from each patient for the use of HFF, and the ethical review committee of the hospital (No. 060623) approved the study design.

Sample Extraction for 2-D Gel Electrophoresis

HFF (50 μL) was precipitated in cold 80% ethanol or 10% trichloroacetic acid (TCA) for 30 min at 4°C, and samples were centrifuged at 13,000 rpm for 30 min. Pellets were immediately resuspended in solubilization buffer (8 M urea, 4% CHAPS, 65 mM DTT, and 40 mM Tris) and vortexed for 1 min after removing residual ethanol or TCA in the pellet. Each sample was freeze-thawed four times using liquid nitrogen prior to electrophoresis.

2-Dimensional Gel Electrophoresis

Proteins were separated by charge in the first dimension using isoelectric focusing (IEF)[26]. HFF proteins (500 μg) were solubilized in a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTE, 0.5% ampholytes (pH 4-7), and bromophenol blue. Immobiline Dry-Strips (Amersham-Pharmacia) covering a pH range of 4-7 were rehydrated in 400 μL of this protein solution for 30 h in low-viscosity paraffin oil. IPG strips containing 500 μg HFF protein were subjected to IEF in a Pharmacia Multiphor II gel apparatus at 20°C. The following voltage/time profile was used: linearly increasing gradient from 0 to 500 V at 1,000 V/h, 500 V at 2,000 V/h, linearly increasing gradient from 500 to 3,500 V at 10,000 V/h, and a final phase of 3,500 V at 35,000 V/h up to a total amount of 48,000 V/h. After isoelectric focusing, the individual strips were incubated in equilibration solution A (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 4% SDS, and 3.5 mg/mL DTT) and then incubated in solution B (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 4% SDS, and 45 mg/mL iodoacetamide) for 15 min each. After equilibration, proteins were separated by size (MW) in the second dimension using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 12 W/gel. Strips were sealed on the top of the gels using sealing solution (1% agarose, 0.4% SDS, 0.5 M Tris-HCl). The running buffer system was the standard Laemmli buffer for SDS-PAGE.

Staining and Destaining

The gels were stained with colloidal Coomassie G-250 (17% ammonium sulfate, 3% phosphoric acid, 0.1% Coomassie G-250, and 34% methanol). After staining, detection was enhanced by placing the gel into 1% acetic acid. The gels were destained with 5 % acetic acid.

Image Analysis

The stained gels were scanned using a UMAX scanner, and data were analyzed using Image Master 2D Elite software (Amersham Pharmacia Biotech, Uppsala, Sweden). Spot detection and matching analysis were performed on each gel, and spots that appeared on every image within the match-set were used as landmarks. These were generally distributed in a+

shape across the sample area of each image to allow for good alignment in both the horizontal and vertical directions. Land-marking aligned the image so that corresponding proteins were matched to each other.

In-gel Digestion

Proteins were subjected to in-gel trypsin digestion[27]. Excised gel spots were destained with 100 mL of destaining solution (50% MeOH in 10% acetic acid) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with 100 mL acetonitrile and dried in a vacuum centrifuge. The dried gel pieces were rehydrated with 20 mL of 50 mM ammonium bicarbonate containing 0.2 mg modified trypsin (Promega) for 45 min on ice. The solution was removed, and 30 mL of 50 mM ammonium bicarbonate were added. The digestion was performed overnight at 37˚C. The peptide solution was desalted using a C18 nano column.

MALDITOFMS Analysis

Custom-made chromatographic columns were used for desalting and concentration of the peptide mixtures prior to MS analysis. A column consisting of 100-300 nL of Poros reverse phase R2 material (20-30 μm bead size, PerSeptive Biosystems) was packed in a constricted GELoader tip (Eppendorf, Hamburg, Germany). A 10-mL syringe was used to force liquid through the column by applying a gentle air pressure. Thirty microliters of the peptide mixture from the digestion supernatant were diluted with 30 μL 5% formic acid, loaded onto the column, and washed with 30 μL of 5% formic acid. Digested peptides were separated by MALDI-TOF.

Protein Identification and Sequence Processing

Tryptic peptides derived from protein spots were analyzed and amino acid sequences were deduced using a peptide *de novo* sequencing program, PepSeq. We searched for identical known protein sequences from NCBInr and EST databases using the MASCOT search program (www.matrixscience.com) and BLAST in order to identify the proteins.

Acknowledgements

This work was partly supported by Korea Ministry of Environment as “The Eco-technopia 21 project” to M.Y. Lee. The 2-dimensional gel electrophoresis system used in this study was provided by SoonChun Hyang University (2007).

References

1. Nayudu, P. L. *et al*. An analysis of human oocytes and follicles from stimulated cycles: oocyte morphology and associated follicular fluid characteristics. *Hum Reprod* **4**:558-567 (1989).
2. Kawano, Y. *et al*. Production of macrophage inflammatory protein-3alpha in human follicular fluid and cultured granulosa cells. *Fertil Steril* **82**:1206-1211 (2004).
3. Schweigert, F. J. *et al*. Peptide and protein profiles in serum and follicular fluid of women undergoing IVF. *Hum Reprod* **21**:2960-2968 (2006).
4. Hanrieder, J., Nyakas, A., Naessén, T. & Bergquist, J. Proteomic analysis of human follicular fluid using an alternative bottom-up approach. *J Proteome Res* **7**: 443-449 (2008).
5. Angelucci, S. *et al*. Proteome analysis of human follicular fluid. *Biochim Biophys Acta* **1764**:1775-1785 (2006).
6. Lee, H. C. *et al*. Identification of new proteins in follicular fluid from mature human follicles by direct sample rehydration method of two-dimensional polyacrylamide gel electrophoresis. *J Korean Med Sci* **20**: 456-460 (2005).
7. Kim, Y. S. *et al*. Proteomic analysis of recurrent spontaneous abortion: Identification of an inadequately expressed set of proteins in human follicular fluid. *Proteomics* **6**:3445-3454 (2006).
8. Anahory, T. *et al*. Identification of new proteins in follicular fluid of mature human follicles. *Electrophoresis* **23**:1197-1202 (2002).
9. Ali, A., Benkhalifa, M. & Miron, P. *In-vitro* maturation of oocytes: biological aspects. *Reprod Biomed Online* **13**:437-446 (2006).
10. Zhou, H. *et al*. Involvement of follicular basement membrane and vascular endothelium in blood follicle barrier formation of mice revealed by ‘*in vivo* cryotechnique’. *Reproduction* **134**:307-317 (2007).
11. Hamel, M. *et al*. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Hum Reprod* **23**:1118-1127 (2008).
12. Jones, G. M. *et al*. Gene expression profiling of human oocytes following *in vivo* or *in vitro* maturation. *Hum Reprod* **23**:1138-1144 (2008).
13. Nan, R., Gor, J. & Perkins, S. J. Implications of the progressive self-association of wild-type human factor H for complement regulation and disease. *J Mol Biol* **375**:891-900 (2008).
14. De Córdoba, S. R. & De Jorge, E. G. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clin Exp Immunol* **151**:1-13 (2008).
15. Josic, D. *et al*. Proteomic characterization of inter-alpha inhibitor proteins from human plasma. *Prote-*

omics **6**:2874-2885 (2006).
16. Garantziotis, S. *et al*. Inter-alpha-trypsin inhibitor attenuates complement activation and complement-induced lung injury. *J Immunol* **179**:4187-4192 (2007).
17. Irving-Rodgers, H. F. & Rodgers, R. J. Extracellular matrix of the developing ovarian follicle. *Semin Reprod Med* **24**:195-203 (2006).
18. Richards, J. S. Ovulation: new factors that prepare the oocyte for fertilization. *Mol Cell Endocrinol* **234**:75-79 (2005).
19. Tejler, L. & Grubb, A. O. A complex-forming glycoprotein heterogeneous in charge and present in human plasma, urine, and cerebrospinal fluid. *Biochim Biophys Acta* **439**:82-94 (1976).
20. Wakui, H. *et al*. High-yield purification of the complex-forming glycoprotein in urine from normal and abnormal subjects. *Clin Chem* **35**:577-581 (1989).
21. Rehman, I. *et al*. Proteomic analysis of voided urine after prostatic massage from patients with prostate cancer: a pilot study. *Urology* **64**:1238-1243 (2004).
22. Tzanavari, T., Bing, C. & Trayhurn, P. Postnatal expression of zinc-alpha2-glycoprotein in rat white and brown adipose tissue. *Mol Cell Endocrinol* **279**:26-33 (2007).
23. Qu, F. *et al*. The role of Zn-alpha2 glycoprotein in sperm motility is mediated by changes in cyclic AMP. *Reproduction* **134**:569-576 (2007).
24. Magi, B. *et al*. Bronchoalveolar lavage fluid protein composition in patients with sarcoidosis and idiopathic pulmonary fibrosis: a two-dimensional electrophoretic study. *Electrophoresis* **23**:3434-3444 (2002).
25. Tsangaris, G. *et al*. The amniotic fluid cell proteome. *Electrophoresis* **26**:1168-1173 (2005).
26. Son, B. S. *et al*. Toxicoproteomic analysis of differentially expressed proteins in rat liver by DEHP. *Mol Cell Toxicol* **3**:299-305 (2007).
27. Jeon, Y. M., Ryu, J. C. & Lee, M. Y. Proteomic analysis of differentially expressed proteins in human lung cells following formaldehyde treatment. *Mol Cell Toxicol* **3**:238-245 (2007).