**CLINICAL PROTEOMICS**

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# Proteomic analysis of human follicular fluid from fertile women

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## Abstract

**Background:** Follicular fluid is a unique biological fluid in which the critical events of oocyte and follicular maturation and somatic cell-germ cell communication occur. Because of the intimate proximity of follicular fluid to the maturing oocyte, this fluid provides a unique window into the processes occurring during follicular maturation. A thorough identification of the specific components within follicular fluid may provide a better understanding of intrafollicular signaling, as well as reveal potential biomarkers of oocyte health for women undergoing assisted reproductive treatment. In this study, we used high and low pH HPLC peptide separations followed by mass spectrometry to perform a comprehensive proteomic analysis of human follicular fluid from healthy ovum donors. Next, using samples from a second set of patients, an isobaric mass tagging strategy for quantitative analysis was used to identify proteins with altered abundances after hCG treatment.

**Results:** A total of 742 follicular fluid proteins were identified in healthy ovum donors, including 413 that have not been previously reported. The proteins belong to diverse functional groups including insulin growth factor and insulin growth factor binding protein families, growth factor and related proteins, receptor signaling, defense/immunity, anti-apoptotic proteins, matrix metalloprotease related proteins, and complement activity. In a quantitative analysis, follicular fluid samples from age-matched women undergoing in vitro fertilization oocyte retrieval were compared and 17 follicular fluid proteins were found at significantly altered levels (p < 0.05) between pre-hCG and post-hCG samples. These proteins belong to a variety of functional processes, including protease inhibition, inflammation, and cell adhesion.

**Conclusions:** This database of FF proteins significantly extends the known protein components present during the peri-ovulatory period and provides a useful basis for future studies comparing follicular fluid proteomes in various fertility, disease, and environmental exposure conditions. We identified 17 differentially expressed proteins after hCG treatment and together these data showed the feasibility for defining biomarkers that illuminate how the ovarian follicle microenvironment is altered in various infertility-related conditions.

**Keywords:** Follicle development, Follicular fluid, Human, Proteomics

## Introduction

The biologic niche where oocyte growth and maturation occurs within the ovary is termed the ovarian follicle. The maturing ovarian follicle is separated from other structures within the ovary by a basement membrane and has both somatic cell components (mural and cumulus granulosa cells) as well as the germ cell component (oocyte). During follicular development there is a coordination of development between the somatic cells and the oocyte. This regulation and coordination involves endocrine, as well as paracrine and autocrine signaling within the specialized microenvironment of the human ovarian follicle. As a follicle is undergoing maturation through the secondary to antral stages, it develops a fluid filled cavity termed the antrum. The antrum of the developing follicle is filled with fluid (termed follicular fluid) which is a select ultrafiltrate of plasma that has been modified by secretion and uptake of specific components by the cells within the follicle itself [1,2]. The ability of proteins to cross this blood-follicle barrier is based both on molecular weight as well as charge characteristics [3]. Follicular fluid is the

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medium by which signaling mediators are transported in and out of the follicle, as well as within the follicle between various cell types. Given that intrafollicular communication is critical for normal oocyte development and reproduction, much effort has been directed at better understanding intrafollicular signaling. It is evident that there is communication from the mural granulosa cells to the cumulus complex [4-6]; from the cumulus complex to the oocyte; and from the oocyte back to the somatic compartment [7-10]. These signaling events can be mediated by soluble small molecules via gap junctions or lipid signals (e.g., cGMP, ffMAS, sphingosine-1-p) [11-14], but the majority of the components for paracrine intrafollicular signaling identified to date involve peptide hormones [15-19].

Because of the intimate proximity of follicular fluid to the maturing oocyte, this biologic fluid provides a unique window into the processes occurring during follicular maturation. The specific components within follicular fluid will help us better understand intrafollicular signaling, as well as reveal potential biomarkers of oocyte health for women undergoing assisted reproductive ART treatment. In this study we used a mass spectrometry approach to identify the proteins present in human follicular fluid (FF) to better understand the paracrine signals at play in the peri-ovulatory time period. Prior mass spectrometry studies have been performed on FF samples obtained from IVF patients. Studies have evaluated fluid from presumably healthy women (such those with male factor infertility) [20,21]; from women who have had successful IVF compared to those who did not [22]; in mature versus immature follicles [23]; and in pathologic states such as infertility [24-26] or in women suffering from repetitive pregnancy loss [27]. In order to develop a better understanding of this matrix, we set out to explore the proteome of healthy young fertile women to better define the normal repertoire of the intrafollicular environment during oocyte maturation. In this study we assayed FF from anonymous oocyte donors undergoing IVF oocyte retrieval, with the hypothesis that the proteins identified will allow for a better understanding the follicular fluid milieu in normal healthy reproductive age women. Human chorionic gonadotropin (hCG) is routinely used as a single injection to induce the final stage of follicle and oocyte maturation during IVF treatment. Therefore, we have also performed a comparison of the follicular fluid proteome from pre-hCG and post-hCG follicular fluid samples to reveal which proteins are significantly changed during follicular maturation.

## Results and discussion

Proteomic analysis of biological fluids is complicated by the large dynamic range of protein concentrations, spanning ten orders of magnitude, greatly exceeding that of any methods used for proteomic analysis [28]. Follicular fluid is a plasma filtrate with the concomitant large dynamic range of protein concentrations that make detection of lower abundance proteins, where new clinically useful marker proteins might be found, challenging. To maximize the depth of coverage, individual follicular fluid samples from 3 ovum donors were immunodepleted of the 14 most abundant plasma proteins and extensively fractionated using alkaline pH reverse phase chromatography of peptides prior to LC-MS/MS analysis. A total of 742 distinct follicular fluid proteins were detected, with 305 of these found in all three samples (Figure 1). A list of the follicular fluid proteins detected in each sample, with score, Swiss-Prot accession numbers, and gene names is presented in Additional file 1: Table S1.

Several groups have previously analyzed post-hCG FF by using mass spectrometry-based methods [9,21-26,29,30]. Twigt et al. identified 246 proteins from FF by using SDS-PAGE and isoelectric focusing (IEF) fractionation prior to LC-MS/MS [31]. A comparison of the combined list of proteins obtained using both fractionation methods, shows that the majority of proteins, 189/246 were also detected in this study (Additional file 1: Table S1). Using immunodepletion, SDS-PAGE, IEF, and strong cation exchange separation strategies, Ambekar et al. recently reported 480 FF proteins [21]. The FF proteome described here includes 297 of those proteins (Additional file 1: Table S1). We have extended the follicular fluid proteome with an additional 413 high confidence distinct proteins. We detected FF proteins not previously reported using MS methods, e.g., cell migration-related MEMO1, S100-A7, and secretogranin-1 and −2.
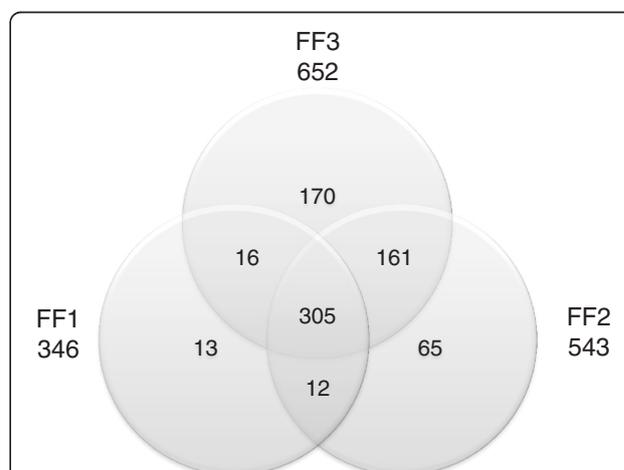


**Figure 1 Overlap of proteins identified at 5% false discovery rate in three follicular fluid samples from ovum donors undergoing ovarian stimulation for assisted reproductive technology.** A total of 742 proteins were identified, of which 305 were found in all three samples. Source accessions for the identified proteins are listed in Additional file 1: Table S1.

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Plasma proteins contribute to the composition of all body fluids, e.g., peritoneal fluid, urine, synovial fluid, saliva, and cerebrospinal fluid. (reviewed in [32,33]) The constituents and concentrations of plasma-derived proteins in body fluids are dependent on the molecular weight, charge, solubility, microvascular permeability and the molecular structure of the compartment. A comparison of the FF proteome to the list of 1929 high confidence human plasma proteins combined from 91 experiments [34] showed 585 proteins in common (Additional file 1: Table S1), a not unexpected finding given the plasma-filtrate origin of the follicular fluid. The coagulation factors are potentially correlated to the inflammatory-related peptides present in FF and have relevant roles within the follicle [35,36]. Thrombin, which found in lower levels in FF as compared to serum [37], was recently shown to be an intra-ovarian signal for optimal follicular luteinization in mice [38]. Antithrombin was found in decreased levels in FF from IVF patients with successful outcomes [22]. To investigate the differences in the FF proteome, which is compartmentalized by a basement membrane to that of an extracellular fluid where there is no barrier, we compared the proteins to a recently published synovial fluid (SF) proteome [39]. Of the 575 SF proteins reported, 321 (56%) were also detected in FF and the overwhelming majority of these (308) were detected in plasma (Additional file 1: Table S1).

The FF proteome was analyzed to determine gene ontology annotations for biological processes, molecular functions, and cellular compartment. The majority of the follicular fluid proteins detected are involved in metabolic processes (19%), cellular processes (14%), cellular communication (11%), and immune responses (11%) (Figure 2A). A significant number are involved in response to stimulus and developmental processes. The top molecular function categories (Figure 2B) were catalytic activity (31%), binding (29%), and receptor activity (15%). Classification based on the subcellular localization (Figure 2C) indicated that 56% of proteins were extracellular.
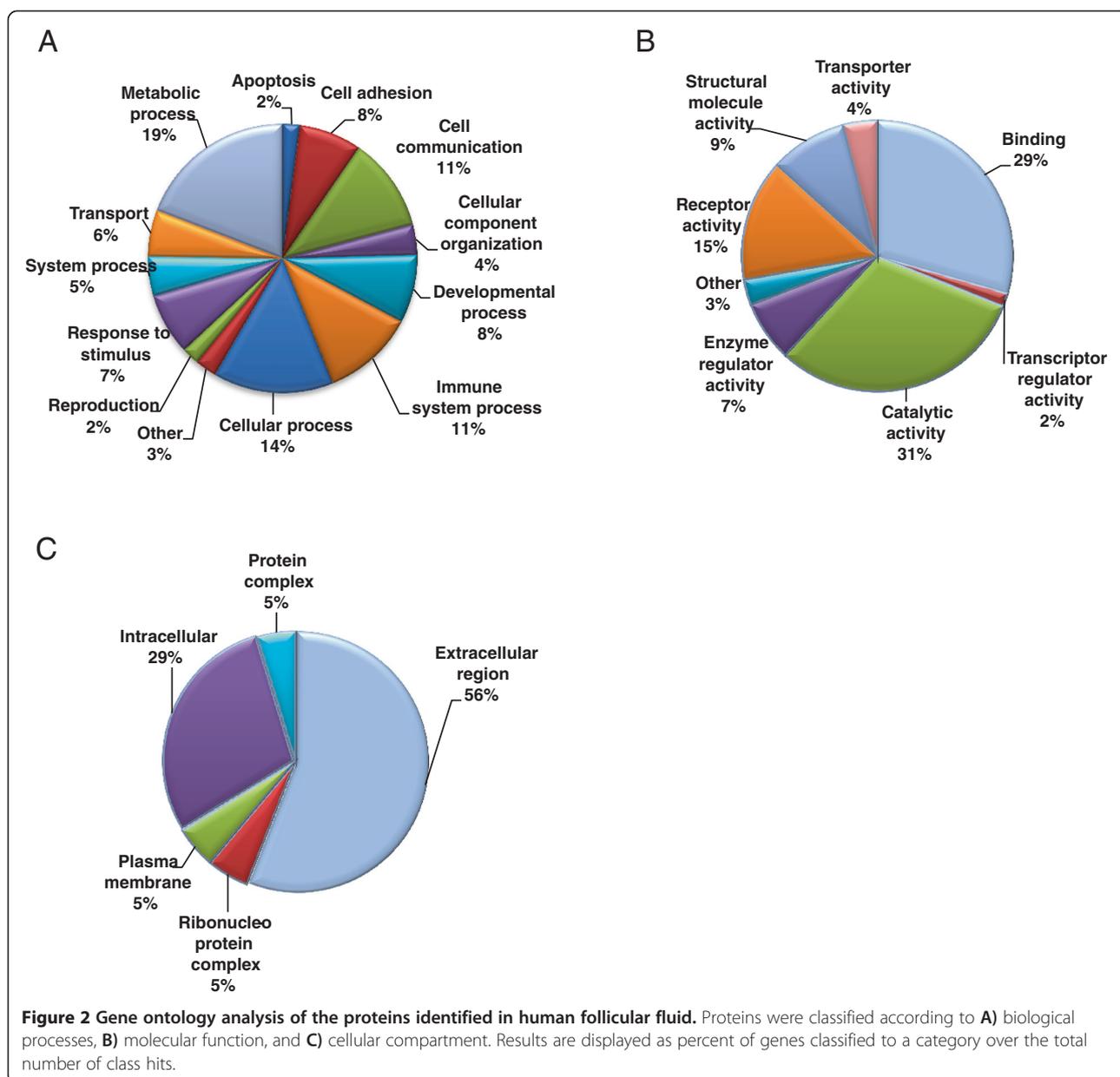
Multiple proteins associated with the inflammatory response were detected: the complement proteins, interleukin-related peptides, proteins regulating chemoattraction (attractin, macrophage migration inhibitory factor, collectin-11), and serine proteases (serine protease-1, 2, and 23). Activation of the inflammatory cascade is required for normal ovulation events, and inhibitors of inflammation such as COX-2 inhibitors have been shown to disrupt ovulation [40,41]. Therefore it is not surprising that several of the proteins in follicular fluid during the peri-ovulatory time are related to inflammation [42]. The super family of serine protease inhibitors (serpins) are involved in coagulation, fibrinolysis, and inflammation [43]. Prior studies, primarily using ELISA methods, have described numerous cytokines and chemokines present in human follicular fluid form patients undergoing IVF, although their relationship to IVF success is debatable [44,45]. In a recent publication, Bianchi et al. [46], using 2-DE and LC-MS for proteomic analysis of follicular fluid from women undergoing IVF, reported inflammation being the predominant class of proteins identified.

A complex program of signaling events is required for follicular maturation [47,48]. Multiple FF proteins with roles in signaling were identified, including insulin growth factor (IGF) and IGF binding proteins, growth factor or growth factor related proteins, anti-apoptotic proteins, and matrix metalloprotease related proteins (Table 1). The intra-ovarian IGF system has been extensively studied in terms of its effects on folliculogenesis and steroidogeneisis (reviewed in [49]). Matrix metalloproteases (MMPs) play a role in the processing of hormones to their active forms, as well as in a variety of other processes including extracellular matrix remodeling and inflammation. MMP-9 has been suggested to be a correlated with IVF success [50]. TIMP1 has been detected in human luteinized granulosa cells, as well as in the ovarian cells from other species [51-53]. Apoptosis of cumulus cells in the oocytes has recently been associated with competence [54].

In addition to plasma-derived proteins, another source of proteins within the follicular fluid is the somatic cells within the follicle, which make up the bulk of the protein constituent of the follicle. Many of the proteins detected were intracellular and may represent events such as cumulus separation from the wall in the mature peri-ovulatory follicle and proteolysis of the follicle wall in preparation for ovulation could release intracellular components. We note that multiple intracellular protein fragments are routinely identified from human plasma [34,42,55,56] and FF [21,31]. The cumulus matrix undergoes expansion concomitant with considerable extracellular matrix remodeling at the peri-ovulatory time period. This is congruous with the presence of several extracellular matrix components, e.g. (laminin subunits, ECM1, Fibrillin-1, CGAT1) present at significant levels in the follicular fluid. Furthermore, many of these proteins are known to be abundantly secreted by granulosa cells in the periovulatory time as the cumulus cells begin to expand and separate in a hyaluronan matrix in preparation for ovulation and fertilization. The proper expansion of this matrix is critical for normal sperm penetration and fertilization events to occur [57].

The maturing oocyte, although the largest cell in the human body, contributes negligibly to the bulk of the secreted protein in the follicle fluid. For example, two key oocyte secreted factors (OSF), growth differentiation factor 9 (GDF-9) and bone morphogenic protein 15 (BMP-15) [8] were not detected. This inability to detect

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**Figure 2 Gene ontology analysis of the proteins identified in human follicular fluid.** Proteins were classified according to **A)** biological processes, **B)** molecular function, and **C)** cellular compartment. Results are displayed as percent of genes classified to a category over the total number of class hits.

oocyte-specific secreted proteins using untargeted mass spectrometry methods [20-26,29-31,58], including our own analyses, likely reflects the much lower abundance of oocyte secreted factors in the follicular fluid relative to the other proteins. Targeted proteomics approaches with enhanced the sensitivity have the potential to detect OSFs present at the tens of ng/mL level [59-61].

Numerous ovarian proteins were detected including follistatin, folllistatin-related proteins 3 and 4, inhibin -alpha, –betaA, –betaB, –betaC, oviduct specific glycoprotein, secreted protein acidic and rich in cysteine (SPARC), pappalysin, and out at first protein homolog (OAF). Some of these were detected for the first time at the protein level in human follicular fluid (Additional file 1: Table S1). The

calcium-binding SPARC protein is expressed in elevated levels in tissues undergoing remodeling and wound repair. SPARC has been shown to be significantly increased in luteinized granulosa cells and is regulated by pro-angiogenic and extracellular matrix factors during the folliculo-luteal transition [62]. Pappalysin has also been detected in serum, and in a recent analysis was reported at elevated levels in the serum of women suffering from preeclampsia compared to normotensive women [63].

Identification of putative markers at the protein level paves the way for rapid tests of oocyte competence, but sensitivity is key to the success of these approaches. We compared the proteins found in FF with microarray data acquired from floating granulosa (predominantly mural

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**Table 1 Follicular fluid proteins with functional roles in signaling**

| Accession | Gene Name | Protein Name |
|---|---|---|
| **IGF related** | | |
| P35858 | IGFALS | Insulin-like growth factor-binding protein complex acid labile subunit |
| P05019 | IGF1 | Insulin-like growth factor I |
| P01344 | IGF2 | Insulin-like growth factor II |
| P08833 | IGFBP1 | Insulin-like growth factor-binding protein 1 |
| P18065 | IGFBP2 | Insulin-like growth factor-binding protein 2 |
| P17936 | IGFBP3 | Insulin-like growth factor-binding protein 3 |
| P22692 | IGFBP4 | Insulin-like growth factor-binding protein 4 |
| P24593 | IGFBP5 | Insulin-like growth factor-binding protein 5 |
| P24592 | IGFBP6 | Insulin-like growth factor-binding protein 6 |
| Q16270 | IGFBP7 | Insulin-like growth factor-binding protein 7 |
| Q13219 | PAPPA | Pappalysin-1 |
| **Metalloproteinase related** | | |
| P08253 | MMP2 | 72 kDa type IV collagenase |
| P14780 | MMP9 | Matrix metalloproteinase-9 |
| Q9UHI8 | ADAMTS1 | A disintegrin and metalloproteinase with thrombospondin motifs 1 |
| Q76LX8 | ADAMTS13 | A disintegrin and metalloproteinase with thrombospondin motifs 13 |
| Q6UY14 | ADAMTSL4 | ADAMTS-like protein 4 |
| Q96KN2 | CNDP1 | Beta-Ala-His dipeptidase |
| P15169 | CPN1 | Carboxypeptidase N catalytic chain |
| P01033 | TIMP1 | Metalloproteinase inhibitor 1 |
| P16035 | TIMP2 | Metalloproteinase inhibitor 2 |
| O95980 | RECK | Reversion-inducing cysteine-rich protein with Kazal motifs |
| **Anti-apoptotic** | | |
| P99999 | CYCS | Cytochrome c |
| P81605 | DCD | Dermcidin |
| Q9Y4L1 | HYOU1 | Hypoxia up-regulated protein 1 |
| P02750 | LRG1 | Leucine-rich alpha-2-glycoprotein |
| P83110 | HTRA3 | Probable serine protease HTRA3 |
| P49908 | SEPP1 | Selenoprotein P |
| **Other growth factor & related** | | |
| P15514 | AREG | Amphiregulin |
| Q9Y5C1 | ANGPTL3 | Angiopoietin-related protein 3 |
| P01019 | AGT | Angiotensinogen |
| O94985 | CLSTN1 | Calsyntenin-1 |
| Q16627 | CCL14 | C-C motif chemokine 14 |
| P26992 | CNTFR | Ciliary neurotrophic factor receptor subunit alpha |
| Q96HD1 | CRELD1 | Cysteine-rich with EGF-like domain protein 1 |
| Q9UBP4 | DKK3 | Dickkopf-related protein 3 |
| Q13822 | ENPP2 | Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 |
| Q8N441 | FGFRL1 | Fibroblast growth factor receptor-like 1 |
| Q9Y625 | GPC6 | Glypican-6 |
| P10912 | GHR | Growth hormone receptor |
| Q04756 | HGFAC | Hepatocyte growth factor activator |

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**Table 1 Follicular fluid proteins with functional roles in signaling** (Continued)

| | | |
|---|---|---|
| P08581 | MET | Hepatocyte growth factor receptor |
| P26927 | MST1 | Hepatocyte growth factor-like protein |
| P07333 | CSF1R | Macrophage colony-stimulating factor 1 receptor |
| P10721 | KIT | Mast/stem cell growth factor receptor |
| Q7Z7M0 | MEGF8 | Multiple epidermal growth factor-like domains protein 8 |
| O14786 | NRP1 | Neuropilin-1 |
| P30086 | PEBP1 | Phosphatidylethanolamine-binding protein 1 |
| P36955 | SERPINF1 | Pigment epithelium-derived factor |
| Q99435 | NELL2 | Protein kinase C-binding protein NELL2 |
| Q9HCB6 | SPON1 | Spondin-1 |
| Q03167 | TGFBR3 | Transforming growth factor beta receptor type 3 |
| Q15582 | TGFBI | Transforming growth factor-beta-induced protein ig-h3 |
| P35590 | TIE1 | Tyrosine-protein kinase receptor Tie-1 |
| P30530 | AXL | Tyrosine-protein kinase receptor UFO |
| P35916 | FLT4 | Vascular endothelial growth factor receptor 3 |
| Q6EMK4 | VASN | Vasorin |

granulosa) and cumulus granulosa cells isolated at the time of oocyte retrieval to determine whether we could detect cellular proteins that are secreted or shed [64]. Communication between the oocyte and the surrounding somatic cells is essential for acquisition of competence (reviewed in [65,66]). A total of 586 FF proteins were found at the transcript level in these cell types (Additional file 1: Table S1). Sixty-three showed increased mRNA expression in the cumulus cells (>1.5 fold), while 55 transcripts showed increased expression in the mural cell transcriptome (>1.5 fold). Thus, proteomic approaches such as this one have sufficient sensitivity to detect protein abundance changes in FF that may arise from the mural and/or cumulus granulosa cell constituents of the follicle.

To quantify differences in protein abundances after hCG treatment, FF proteins from 6 individuals (3 pre-hCG treatment and 3 post-hCG treatment were compared using iTRAQ mass tags to measure relative abundance changes. The ratios from each group were compared using Student's *t*-test and 17 proteins were found to be significantly different (p < 0.05). Eleven proteins were significantly higher in the post-hCG samples compared to the pre-hCG samples, and six proteins were significantly lower in the post-hCG samples compared to the pre-hCG samples (Table 2). Prior studies have shown that the majority of these proteins have been detected in FF by orthogonal detection methods such as Western blotting or ELISA. For example prostatic acid phosphatase, metalloproteinase inhibitor 1, alpha-2-HS-glycoprotein, gelsolin, prothrombin, coagulation factor X, inhibin alpha, histidine-rich glycoprotein and extracellular matrix protein 1 have been detected in human follicular fluid by

orthogonal methods [67-73]. Further, 10 of the 17 proteins had transcripts that were present in both mural and cumulus granulosa cells (Additional file 1: Table S1). Alpha-2-HS-glycoprotein, N-acetylmuramoyl-L-alanine amidase, prothrombin, pantetheinase, complement component C8 alpha chain, Beta-Ala-His dipeptidase and histidine-rich glycoprotein are presumably not of granulosa cell origin based on the transcriptome comparison. This provides important validation of the iTRAQ proteins detected, although a limitation of our study is that the levels of changes could not be validated within our own samples due to sample volume limitations. The proteins showing differences belonged to a variety of functional processes, including protease inhibition, inflammation, angiogenesis, and cell adhesion. Complement C8 alpha chain was found in higher abundance (1.4-fold) in pre-hCG FF. The role of the complement factors in follicle maturation is unclear, though it has been postulated that they are possible oocyte maturation factors [74]. Another complement factor, C3, was differentially expressed in women with severe ovarian hyperstimulation syndrome [69].

TIMP-1 has been shown to be expressed in luteinized granulosa cells of humans [52], and was found in 4.4-fold higher levels in post-hCG samples. MMPs and tissue inhibitor of metalloproteinases (TIMPs) have been reported to have multiple effects on ovarian function, particularly remodeling the extracellular matrix [75]. A study in endometriosis patients undergoing IVF showed that mature follicles yielding MII oocytes had significantly higher TIMP1 levels compared to follicles yielding immature germinal vesicle oocytes, and that embryos of good morphology were correlated with follicles with higher TIMP1 [73]. Exposure of rats to persistently

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**Table 2 Follicular fluid proteins with altered abundance after hCG treatment**

| Protein | Accession | Gene Name | p value | Relative abundance |
|---|---|---|---|---|
| **Increased abundance post-hCG** | | | | |
| Prostatic acid phosphatase | P15309 | PPAP | 1.1E-06 | 22.0 |
| Metalloproteinase inhibitor 1 | P01033 | TIMP1 | 8.3E-04 | 4.4 |
| Alpha-2-HS-glycoprotein | P02765 | FETUA | 7.5E-03 | 2.4 |
| N-acetylmuramoyl-L-alanine amidase | Q96PD5 | PGRP2 | 3.1E-03 | 2.4 |
| Complement component C7 | P10643 | CO7 | 1.1E-02 | 2.2 |
| Gelsolin | P06396 | GELS | 1.1E-02 | 2.0 |
| Vitronectin | P04004 | VTNC | 2.2E-02 | 1.8 |
| Prothrombin | P00734 | THRB | 5.0E-03 | 1.7 |
| Carboxypeptidase N subunit 2 | P22792 | CPN2 | 2.6E-02 | 1.7 |
| Cystatin-C | P01034 | CYTC | 4.8E-02 | 1.5 |
| Extracellular matrix protein 1 | Q16610 | ECM1 | 3.5E-02 | 1.5 |
| **Decreased abundance post-hCG** | | | | |
| Pantetheinase | O95497 | VNN1 | 4.7E-02 | 1.3 |
| Complement component C8 alpha chain | P07357 | CO8A | 2.6E-02 | 1.4 |
| Coagulation factor X | P00742 | FA10 | 4.5E-02 | 1.9 |
| Beta-Ala-His dipeptidase | Q96KN2 | CNDP1 | 3.0E-02 | 3.8 |
| Inhibin alpha chain | P05111 | INHA | 1.2E-02 | 4.4 |
| Histidine-rich glycoprotein | P04196 | HRG | 1.9E-02 | 5.5 |

elevated levels of TIMP1, designed to emulate endometriosis, showed an overall negative affect on ovarian function as it disturbed folliculogenesis and lowered the number of corpora lutea (CL) formed [53]. Inhibin alpha was one of the most up-regulated proteins pre-hCG, consistent with prior studies showing significant expression specifically in granulosa cells [76] as well as its known regulation by LH signaling [77,78]. These proteins have the potential to be biomarkers of normal luteinization and, in combination, could be used as a panel to assay follicular health at the folliculo-luteal transition. The secreted protein extracellular matrix protein 1 (ECM1) is a glycoprotein that inhibits the activity of MMP9 [79]. Higher FF and serum MMP9 levels are associated with IVF success [50]. Thus, levels of ECM1 could be a predictor of oocyte quality.

Histidine-rich glycoprotein (HRG) is an abundant plasma protein that binds a variety of ligands including fibrinogen, heparin, and thrombospondin and has roles in angiogenesis, coagulation, and the immune system [80-84]. HRG has been previously reported in FF [71] and was found in our study to be 5.5-fold higher levels in pre-hCG samples. A single nucleotide polymorphism (C633T) in HRG, which results in a serine to proline at position 186, is associated with primary recurrent miscarriage [85] and a lower pregnancy rate in IVF patients [86]. The tryptic fragment encompassing this site is only

3 amino acids in length, thus too short to be detected by the LCMS methods used in this study. The angiogenic factors fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are produced by the pre-ovulatory follicle and may be key regulators of the angiogenic balance in the follicle [87]. Elevated VEGF levels are associated with unfavorable clinical IVF outcomes such as ovarian hyperstimulation syndrome (reviewed in [88]) and dissolved oxygen content of the follicle, which is related to VEGF, has been correlated with poor oocyte quality [89]. HRG negatively regulates VEGF signaling [90], and HRG levels could be a functional link to between VEGF levels [10,77,91] and responses to stimulation and IVF outcome.

One of the most down-regulated proteins pre-hCG was prostatic acid phosphatase. Lower levels of this protein in follicle fluid are associated with immature oocytes (as assessed by fertilization) [70], in accordance with the levels seen in pre-hCG follicles. Furthermore, in addition to lower levels of prostatic acid phosphatase from pre-HCG FF, we also found lower levels of prothrombin and PGRP2. Recently, Severino et al., [92] used a similar MS approach in FF from IVF patients who conceived versus those who did not and found these three proteins were more abundant in successful IVF. This is consistent with our findings, since pre-hCG follicles do not contain oocytes competent for fertilization. Potentially, a panel of

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proteins that are regulated during hCG administration would yield a protein signature of optimal luteinization for an individual follicle.

This comparative proteomics analysis provided information about abundance changes in follicular phase fluid (pre-hCG) vs. at the luteal transition (post-hCG), to our knowledge the first such analyses using pre-hCG follicular fluid. Our comparative proteomics analysis showed relatively few significant differences in protein expression from pre- versus post-hCG follicular fluid despite the knowledge that several proteins related to intra-follicular signaling are known to either be up-or down-regulated at the time of the LH surge (e.g., EGF-like growth factors, C-type natriuretic peptide [6,93]). The reason for this is that these proteins were below the detection limit of the iTRAQ portion of our study. However, our unbiased mass spectrometry analysis did detect the EGF-like growth factor amphiregulin, which is present at the average level of 30 ng/ml in post-hCG samples [93]. Therefore it is likely that a combination of sample heterogeneity and preferential detection of higher abundance proteins were the reasons for the relatively low number of iTRAQ differences seen. Our analysis was performed on follicle fluid obtained from ovum donors after the granulosa cells were exposed to an artificial luteinization signal from exogenous hCG administration. Given inter-patient variability, it would be of great benefit to be able to compare this to unluteinized follicle fluid from within the same patients (ideally with serum samples as controls) to allow for enhanced detection of pathways specifically activated by the LH surge and to better understand the intrafollicular environment during the earlier stages of follicle development. Of the FF proteins found to be significantly different between pre-hCG and post-hCG samples, some have putative or known functions in follicle maturation. Others may represent biomarkers of follicle maturation, although their functional role, if any, remains uncertain. The proteins described here will provide a framework for potentially assessing the competence of an oocyte from a given follicle.

## Conclusions

This analysis has been the most comprehensive proteomic evaluation of human follicular fluid in a single study to date, with 742 distinct proteins identified. This extends the FF proteome to 982 high confidence proteins, underscoring the utility of multiple, orthogonal protein and peptide methodologies for comprehensive examination of this complex proteome. This database of FF proteins provides a useful basis for studies comparing follicular fluid proteomes in various fertility, disease, and environmental exposure conditions. In particular, alterations in the protein complement of FF from infertile patients with varying etiologies such as advanced female age, endometriosis, or polycystic ovarian syndrome could be compared to our study on fertile egg donors to improve our understanding of how the ovarian follicle microenvironment is altered under these conditions.

The goal of the present study was to characterize the follicular fluid proteome from fertile women and quantify differences in protein abundances after the hCG administration. A comparison with microarray data from mural granulosa cells and cumulus granulosa cells isolated at the time of oocyte retrieval [64] showed that we could detect many differentially expressed transcripts at the protein level in FF. This ability to detect proteins that may be derived from the somatic cell repertoire of the follicle allows for a functional analysis of these distinct compartments during follicular maturation. Differences in the cumulus cell transcriptome, for instance, have been correlated with IVF outcomes [94-96]. To improve our understanding of normal human intrafollicular dynamics, we performed an untargeted quantitative analysis to reveal biomarker candidates and/or signaling cascades active during follicle growth and maturation. The proteins identified here provide an additional layer of understanding of which proteins are expressed during this time and can provide a method of assessing the somatic compartment of the human follicle. Future directions should focus on targeted protein quantitation from individual follicular aspirates as the basis for noninvasive assessment of oocyte quality. The proteins described here that change in response to hCG may be detected in serum or follicular fluid as markers of premature or appropriate luteinization to allow for improved ovarian stimulation outcomes.

## Methods
### Research approval
All subjects gave consent for donation of follicular fluid for research purposes as part of a UCSF Institutional Review Board approved IVF tissue bank protocol.

### Source and collection of human follicular fluid samples
Patients undergoing assisted reproductive technology (ART) by standard ovarian stimulation protocols were recruited to collect follicular fluid (FF). Details regarding the stimulation parameters, oocyte retrieval and FF preparation and storage have been previously described [93,97]. For all experiments, FF was obtained from mature sized follicles (≥16 mm diameter). For the comprehensive proteomics analysis, follicular fluid from three anonymous ovum donors (all age <30) receiving down-regulated ovarian stimulation protocols were used. Each patient had either one or two aspirates collected at the time of oocyte retrieval (36 hours after hCG treatment for all post-hCG samples) and only a single FF sample from each donor was used for mass spectrometry analysis.

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For relative quantification, pre-hCG FF was obtained from three patients: i) a single ovum donor who did not inject hCG and ii) two stimulated IVF patients with single lead follicles that were aspirated to allow salvage of ovarian stimulation. For the post-hCG samples, FF from three age-matched donors, using the same type of ovarian stimulation protocol, were used.

### Sample preparation for mass spectrometry

The protein concentrations were determined using BCA Assay (Thermo Scientific, San Jose, CA). Follicular fluid samples were processed according to the manufacturer's instructions using a MARS-Hu-14 immunodepletion column from Agilent Technologies (Santa Clara, CA). The protein flow-through fraction was collected and desalted by using a 3 kDa molecular weight cutoff (MWCO) centrifugal concentrator (Sartorius AG, Goettingen, Germany). Immunodepleted samples were denatured using 6 M urea, cysteines reduced with 500 mM dithiothreitol (DTT) and alkylated with 500 mM iodoacetamide (IAA) followed by an over-night incubation with sequencing grade trypsin (Promega, Madison, WI) at 37°C. The resulting peptides were acidified with formic acid and desalted using Oasis HLB Extraction Cartridges (Waters Corporation, Milford, MA). For iTRAQ analysis, a 50 μg aliquot immunodepleted follicular fluid sample from 6 patients (3 pre-hCG and 3 post-hCG treatment, age-matched) was digested with trypsin and labeled with 8plex iTRAQ reagent according to manufacturer's protocol (AB Sciex, Foster City, CA). A pool comprised of equal amounts of protein from each of the post-hCG samples was labeled and used as the reference standard. Both labeled and unlabeled samples were subjected to offline peptide fractionation by using a Paradigm MS4 HPLC System (Michrom, Auburn, CA) equipped with a Zorbax Extend-C18 column (4.6 × 100 mm, 3.5 μm particle size, Agilent Technologies, Santa Clara, CA) and a guard column of the same packing material. A 100 μg sample of protein digest was reconstituted in 100 μL of Solvent A (0.1% $NH_4OH$, pH 10). Peptides were eluted using a linear gradient of 2-50% Solvent B (0.1% $NH_4OH$ in acetonitrile) over 30 min. and 30 fractions were collected. The fractions were vacuum-dried, reconstituted in 0.1% formic acid, and stored at −80°C until analysis.

### LC-MS/MS and bioinformatics

For protein identification, peptides were analyzed by LC-MS/MS using a nanoLC Ultra system (Eksigent Technologies, Dublin, CA) interfaced with a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated using an Acclaim PepMap100 C18 column (75 μm i.d. × 15 cm, 3 μ, 100 Å) with a linear gradient of 2-40% B (98% ACN, 0.1% FA) over 60 minutes. MS data were acquired using an LTQ

Orbitrap Velos with data-dependent ion selection consisting of the initial MS scan (m/z 350–1600) followed by eight MS/MS scans (m/z 100–1600). The .raw files were processed by Mascot Daemon v.2.2.2 (Matrix Science, Boston, MA) to generate mgf files. Data from the iTRAQ labeled samples were acquired using the same LC conditions using a nano2D MDLC (Eksigent) interfaced with a QSTAR Elite mass spectrometer (AB Sciex). The Paragon algorithm in ProteinPilot v.4.2 (AB Sciex, Foster City, CA) was used for protein identification and quantification and false discovery rates (FDRs) assessed by decoy database searching [98]. Data were searched against the SwissProt database v20120222 using the following parameters: carbamidomethylation of cysteines, trypsin enzyme, *Homo sapiens* species filter, and thorough search effort. Proteins detected with 5% local FDR were reported. To determine significant changes in protein abundances log-transformed iTRAQ ratios were analyzed using Student's *t*-test and the threshold for differential abundance was p < 0.05. Gene Ontology analysis of proteins was carried out by using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system [99] and Swiss-Prot KB gene ontology data [100].

### Additional file

**Additional file 1: Table S1.** Proteins identified in human follicular fluid. Proteins detected in human follicular fluid. Proteins detected at 5% FDR in at least one sample were reported. The first column for each sample, FF1-FF3, "Rank" shows the ranking of the specified protein to all other proteins identified in that sample and the second column, "Unused ProtScor" is the ProteinPilot confidence score. Follicular fluid proteins also reported in Ambekar, et al., [21] and Twight et al., [31] are denoted by an "x". Proteins detected for the first time in FF are in bold. Farrah et al., [34] shows FF proteins that are in the Human Plasma PeptideAtlas list and Balakrishnan et al., [39] shows proteins detected in synovial fluid. Follicular fluid proteins were compared to granulosa cell transcriptomic data from Koks et al., [64] and the corresponding FF proteins denoted with an "x". Mural granulosa cells, mGC; cumulus granulosa cells, cGC.

### Abbreviations

ART: Assisted reproductive technology; FF: Human follicular fluid; IGF: Insulin growth factor; IVF: In vitro fertilization.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

AMZ contributed to study conception and design, acquisition of clinical samples, and bioinformatics analysis. MEH performed sample preparation and data analysis. MEA performed sample preparation and proteomics analyses. KEW conducted mass spectrometry data acquisition and bioinformatics analyses. AMZ, KEW, and MEH provided substantial intellectual contributions and participated in manuscript preparation. All authors read and approved the final manuscript.

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