Phosphoproteomics Analysis of Endometrium in Women with or without Endometriosis

Hong-Mei Xu1,2, Hai-Teng Deng2, Chong-Dong Liu1, Yu-Ling Chen3, Zhen-Yu Zhang1

1Department of Obstetrics and Gynecology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China
2Department of Obstetrics and Gynecology, Beijing Fengtai Hospital, Beijing 100071, China
3Ministry of Education Key Laboratory of Bioinformatics, School of Life Sciences, Tsinghua University, Beijing 100083, China

Abstract

Background: The molecular mechanisms underlying the endometriosis are still not completely understood. In order to test the hypothesis that the approaches in phosphoproteomics might contribute to the identification of key biomarkers to assess disease pathogenesis and drug targets, we carried out a phosphoproteomics analysis of human endometrium.

Methods: A large-scale differential phosphoproteome analysis, using peptide enrichment of titanium dioxide purify and sequential elution from immobilized metal affinity chromatography with linear trap quadrupole-tandem mass spectrometry, was performed in endometrium tissues from 8 women with or without endometriosis.

Results: The phosphorylation profiling of endometrium from endometriosis patients had been obtained, and found that identified 516 proteins were modified at phosphorylation level during endometriosis. Gene ontology annotation analysis showed that these proteins were enriched in cellular processes of binding and catalytic activity. Further pathway analysis showed that ribosome pathway and focal adhesion pathway were the top two pathways, which might be deregulated during the development of endometriosis.

Conclusions: That large-scale phosphoproteome quantification has been successfully identified in endometrium tissues of women with or without endometriosis will provide new insights to understand the molecular mechanisms of the development of endometriosis.

Key words: Endometriosis; Endometrium Tissue; Phosphopeptide Enrichment; Phosphoproteome

Introduction

Endometriosis is defined as the presence of functional endometrial epithelium and stroma in areas outside the uterus, most typically inside the pelvis, but occasionally in extra-pelvic sites. It is a progressive, estrogen-dependent gynecologic common disorder in the reproductive aging population, usually characterized by pain symptoms (e.g., dysmenorrhea, dyspareunia, dysuria, dyschezia, or noncyclic pelvic pain), infertility, and ovarian endometrioma (chocolate cyst). The prevalence of this disease is accepted to be around 10–15% of all women of reproductive age.1,2 Annual estimates for direct and indirect costs for endometriosis care exceed $20 billion nationally in the United States in the 1990s.3 The pathology of endometriosis is unknown, and there are several hypotheses have been proposed. Sampson’s theory of retrograde menstruation1,3 has been widely proposed to explain the etiology of endometriosis, but it does not account for the fact that this endometrial cells survive in women with the disease and not in healthy ones. To explain this phenomenon, the hypotheses that eutopic endometrium has been subject to extensive investigation.1,2 These hypotheses proved that eutopic endometrium shows enhanced the ability of proliferation, implantation and angiogenesis, and greater probability of escaping the unfavorable conditions of the ectopic environment.

As one of the most important posttranslational modifications, phosphorylation was estimated to happen on one-third of proteins in eutopic endometrium tissues.

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all proteins and modulates most processes in living cells.\[^{[4]}\] Therefore, the characterization of the phosphorylation sites of proteins within various signaling pathways can enhance the understanding of specific disease pathologies.\[^{[5]}\] The importance of the phosphorylation – dephosphorylation cycle is supported by the presence of a high number of protein kinases and phosphatases in the human genome, which constitute approximately 2% of all genes.\[^{[6]}\] The preferred method for large-scale identification and quantification of phosphorylation sites is liquid chromatography-tandem mass spectrometry (LC–MS/MS). The bottleneck for comprehensive analysis of the phosphoproteomics is that the abundance of phosphorylated proteins is generally low in complex sample mixtures. Until date, advanced methods for the selective enrichment strategy, antiphosphotyrosine antibodies, affinity chromatography including immobilized metal affinity chromatography (IMAC), and titanium dioxide (TiO\(_2\)) chromatography as well as strong cation exchange chromatography are commonly used.\[^{[7]}\] Combining separation techniques with phosphopeptide enrichment can reduce sample complexity and increase coverage.

Both gene expression and proteomic profiling of isolated cell or tissue related to endometriosis populations have been extensively studied and were described in a wealth of literature.\[^{[8–11]}\] Through those studies, endometriosis has been shown to be tightly regulated by the differential activity of different signaling pathways.\[^{[12,13]}\] However, the phosphoproteome of the endometrium in endometriosis, on the other hand, has largely been neglected. Therefore, it is of great importance to study protein phosphorylation on a large-scale to obtain a global picture of signaling events within the endometrium. In this study, we combined already well-established methods,\[^{[14]}\] a TiO\(_2\)-based preenrichment step, to isolate a relatively pure phosphorylated peptide fraction from very complex peptide mixtures. After the preenrichment, the phosphopeptides were eluted and subjected to sequential elution from IMAC (SIMAC), enabling separation of mono-phosphorylated and multi-phosphorylated peptides. Furthermore, this separation was allowed for LC–MS/MS. After filtering, a total of 5825 phosphopeptides corresponding to 502 proteins were detected.

### Methods

**Reagents and materials**

Phosphate-buffered saline (PBS) was purchased from Wisent (Montreal, QC) and used without further purification. Dithiothreitol (DTT) was purchased from Merck (Whitehouse Station, NJ, USA). Sequencing grade-modified trypsin was purchased from Promega (Fitchburg, WI, USA). Iodoacetamide (IAA) and phosphatase inhibitor cocktail were purchased from Sigma (St. Louis, MO, USA). Complete protease inhibitor without ethylenediaminetetraacetic acid was from Roche Applied Science (Meylan, France). Dimethyl sulfoxide was purchased from Applichem (St. Louis, MO, USA). A BCA protein assay kit was purchased from Solarbio (Beijing, China). Benzonase was from Merck (Darmstadt, Germany). Titansphere Phos-TiO Kits were from GL Science (Japan). OASIS HLB sample extraction products were from Water Corporation (Milford, MA, USA). 3M Empore C8 disk was from 3M Bioanalytical Technologies (St. Paul, MN, USA). Water was double deionized. All other reagents used in the experiments were of mass spectrometry grade.

### Human endometrium tissue samples

Human endometrium tissue samples used in this study were obtained from the Department of Obstetrics and Gynecology, Beijing Chao-Yang Hospital, Capital Medical University. Laparoscopy was performed to investigate either cause of ovary cyst, suspected endometriosis, or not. Individuals were divided into two groups: Four women were severe endometriosis group and four women without endometriosis were used as a control. Women with or without endometriosis were diagnosed based on visualization of endometriotic lesions and histopathology criteria. The stage of the endometriosis was assessed according to the revised classification of the American Fertility Society.\[^{[15]}\] There were no significant differences in demographic features between two separate groups. Each patient exhibited regular menses and no anatomical changes in the uterus. Patients would be excluded including a history of tuberculosis peritonitis, using of oral contraception or hormonal therapy or an intra-uterine device in the last 3 months and presumed pregnancy. Through the laparoscopy or histopathology diagnosis, patients would be excluded including pelvic congestion syndrome, pelvic inflammatory, adenomyosis, uterine fibroids, and malignancy. Control women were individuals that had ovary cyst without chronic pelvic pain history and without any pelvic endometriosis determined by laparoscopy. Endometrial samples were snap frozen in liquid nitrogen after being dissected in 4°C PBS and had been stored at −80°C until processing. All biopsy specimens were collected from the middle secretory phase based on the endometrial dating criteria of Noyes et al.\[^{[16]}\] All patients had signed a written informed consent before surgery and had agreed on the collection of tissues for research. The Local Ethical Committee of Beijing Chao-Yang Hospital Affiliated to Capital Medical University had approved the study protocol.

### Tissue fractionation procedure

The study protocol was performed at Ministry of Education Key Laboratory of Bioinformatics, School of Life Sciences, Tsinghua University. Tissue samples were skived in liquid nitrogen and homogenized with glass homogenizer for 10 times in lysis buffer, which is pH 8.0, 8 mol/L urea, supplemented with phosphatase inhibitor cocktail, complete protease inhibitor, and phenylmethylene)sulfonyl fluoride. Every 100 mg tissue sample was used 1 ml lysis buffer. Tissue samples were homogenized by sonicated 2 s after stopping 2 s for 60 times in a lysis buffer. All steps were performed at 4°C during the isolation process. Tissue samples lysis was incubated at 4°C for 40 min and off centered by 4°C centrifuge with 15,000 r/min for 20 min. The supernatant was recovered and aliquoted as protein. Protein concentration which was

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determined by BCA protein assay in the human endometrium samples used in this study ranged from 350 to 775 mg/ml. Protein lysates from 4 patients with severe endometriosis were pooled together to make a quality be 2 mg. Proteins from 4 control patients had been also pooled to obtained 2 mg.

**Proteomic sample preparation**

Proteins of 2 mg were reduced with 10 mmol/L DTT followed by incubation at 55°C for 30 min and alkylated by incubation in 20 mmol/L IAA in darkness for 45 min at ambient temperature. Protein lysates were diluted to a final urea concentration of 1.5 mol/L with 50 mmol/L ammonium bicarbonate and digested with trypsin (substrate: Enzyme = 50) at 37°C overnight. The solvent was acidified using 10% trifluoroacetic acid (TFA) to make pH be 2.0

**Phosphorylated peptide enrichment**

Oasis HLB 1 cc

Deionization: The peptide solution should be desalted using OASIS HLB micro columns. The micro columns were conditioned with 1 ml methanol twice and equilibrated with 1 ml deionized water for 3 times. The samples of two groups were loaded to the tips for 3 times, respectively. The loading HLB micro columns were washed with 1 ml 5% methanol twice and eluted with 200 μl methanol twice. The elution was dried with vacuum centrifugal dryer to left 100 μl.

**Titanium dioxide purification**

The TiO$_2$ tip was equilibrated by 20 μl buffer A (1% TFA 1 ml + 4 ml acetonitrile [ACN]) and 20 μl buffer B (solution B 1 ml + 3 ml buffer A), respectively and centrifuged with 3000 × g at room temperature (RT) for 2 min. Solution B was provided by TiO$_2$ kits. Fifty microliters of a sample and 100 μl of buffer B were added to the tip and mixed with pipetting 3 times. After centrifugation with 1000 × g at RT for 10 min, the sample in the tube was put back into the tip again and centrifuged with 1000 × g at RT for 10 min. The solvent was discarded. The tip was rinsed first with 20 μl of buffer B and centrifuged. The solvent was discarded. The tip was rinsed with 20 μl of buffer A twice and centrifuged. The solvent was discarded. The tip was first eluted with 50 μl of 5% ammonium hydroxide solution and centrifuged. The tip was second eluted with 50 μl of 5% pyridine solution and centrifuged. The two steps elution of phosphopeptides was mixed and dried with vacuum centrifugal dryer to left 10 μl prior to SIMAC.

**Sequential elution from immobilized metal affinity chromatography**

Phosphopeptides were redissolved in wash/equilibration solution (250 mmol/L acetic acid with 30% CAN). The pH was adjusted to 1.6–1.8 using 10% TFA. A total of 60 μl of IMAC slurry was washed twice with wash/equilibration solution prior to incubation with the phosphopeptide solution. The IMAC beads were incubated with the phosphopeptide solution for 30 min at RT under continuous shaking. After incubation, the solution was centrifuged briefly to pellet the beads. The flow through was removed carefully without disturbing the beads and the contents transferred to a new microcentrifuge tube. The IMAC beads were washed with 500 μl of wash/equilibration solution and 500 μl of deionized water separately, which was collected together with the flow through. Mono-phosphorylated and nonphosphorylated peptides were eluted slowly from the IMAC beads using 70 μl of 20% ACN, 1% TFA and collected together with the flow through. The multi-phosphorylated peptides were subsequently eluted from the IMAC material using 80 μl of 1% NH$_4$OH, pH 11.3. The multi-phosphorylated peptide sample was acidified and desalted using stage tip (stop-and-go-extraction tip). The IMAC flow through and the mono-phosphorylated peptide fraction were adjusted to 70% ACN, 1% TFA, and incubated for with the same amount of TiO$_2$ material as used in the TiO$_2$ prepurification. The same steps as used in the TiO$_2$ prepurification were performed, and the phosphopeptides elution was recovered. After elution from TiO$_2$, the samples were acidified using 10% TFA to pH <2 and desalted using stage tip (stop-and-go-extraction tip).

**Liquid chromatography-tandem mass spectrometry**

Peptide digests were analyzed by an EASY-nLCII™ integrated nano-high-performance liquid chromatography system (Proxeon, Denmark), which was directly interfaced with a linear trap quadrupole (LTQ)-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The injections of each sample were resolved on a 75 μm ID × 360 μm OD × 150 mm length × 150 mm length capillary column (Upchurch, Oak Harbor, WA, USA), slurry packed in house with 5 μm, 300 Å pore size C-18 silica-bonded stationary phase (Varian, Lexington, MA, USA). Following precolumn and analytical column equilibration, each sample was loaded onto a 20 mm reversed-phase (C-18) precolumn at 3 μl/min for 6 min with mobile phase A (0.1% formic acid in water). Peptides were eluted at a constant flow rate of 200 μl/min by development of a linear gradient of 0.33% mobile phase B (0.1% formic acid in ACN) per min for 120 min and then to 95% B for an additional 15 min. The column was washed for 15 min at 95% mobile phase B and then equilibrated with 100% mobile phase A for the next sample injection. The LTQ-Orbitrap Velos mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur 2.0.7 software (Thermo Fisher Scientific Inc, USA) and there was a single full-scan mass spectrum in the Orbitrap (m/z 400 to m/z 1800, 30,000 resolution) followed by 20 data-dependent MS/MS scans in the ion trap at 35% normalized collision energy (collision-induced dissociation).

**Data analysis**

All MS raw files were processed with Proteome Discoverer (Version 1.3, Thermo Scientific, USA) by standardized workflows. Briefly, the generated peak list files were searched against a concatenated human FAST-All, (FAST) database. These MS/MS spectra were searched against a composite database of mouse proteins containing sequences first in the forward direction and then in the reverse direction. The enzyme specificity was set to trypsin with the maximum number of missed cleavages set to 2. The precursor mass tolerance was set to 5 parts-per-million (ppm) for the first search (used for nonlinear mass recalibration) fragment mass...
deviation was set to 0.8 Da. Cysteine carbamidomethylation was selected as fixed modification; methionine oxidation, and phosphorylation on serine, threonine, and tyrosine were selected as variable modifications. The false discovery rates for peptides, proteins, and sites were set at 0.01 and peptides with a minimum length of seven amino acids were considered for phosphopeptide identification. Protein groups were identified from tryptic-digested samples based on at least two unique peptides. For protein identification, we used IPI database. A protein group was removed if all identified peptides assigned to this protein group were also assigned to another protein group. Significance was regarded only when the ratio of spectral counts between two groups was more than 2 or <0.5. The differentially expressed phosphoproteome were submitted to the DAVID database (the database for annotation, visualization, and integrated discovery, http://david.abcc.ncifcrf.gov) to be classified into different gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation groups.

RESULTS

Subject characteristics
Eight women complicated with ovary cyst were recruited in this study. They all underwent laparoscopy and diagnosed by histopathology. The characteristics of these women are listed in Table 1.

Phosphoproteome profiling
After in-lysates digestion, the LTQ-Orbitrap Velos mass spectrometer prepared from endometrium tissues contained 2615 phosphopeptides from 501 proteins identified in samples of endometriosis patients and 3137 phosphopeptides from 441 proteins in women without endometriosis. In total, we identified 516 proteins that were significantly differentially expressed (fold-change >2) in two groups revealed 335 up-regulated and 181 down-regulated phosphorylation sites.

Ratio of phosphorylated serine:phosphorylated threonine:phosphorylated tyrosine
The identified 516 proteins contained 3522 phosphorylation sites in endometrium tissues. The ratio of the detected phosphorylated serine (pSer, 53%), phosphorylated threonine (pThr, 33%), and phosphorylated tyrosine (pTyr, 14%) residues in this study was 3.8:2.4:1 (pSer: pThr: pTyr), which is shown in Figure 1.

Analysis of gene ontology
In order to obtain a system-level view of differentially expressed phosphoproteome, all the phosphorylated proteins identified in our analyses were classified in terms of cellular component, biological process, and molecular function through analysis of GO. As shown in the cellular component figure, the majority of these proteins are located in cell and cell part [Figure 2a]. The top five several of biological process categories of phosphoproteins identified in endometrium tissues were: (1) Cellular process (21.36%), (2) Physiological process (19.96%), (3) Metabolism (9.25%), (4) Biological regulation (9.25%), and (5) Regulation of biological process (8.05%) [Figure 2b]. Among the identified phosphoproteins, the relative proportions of the corresponding proteins with functions relating to binding, catalytic activity, and enzyme regulator activity were 45.76%, 30.08%, and 7.2%, respectively [Figure 2c].

Analysis of Kyoto Encyclopedia of Genes and Genomes pathways
Specific pathways are important for disease progression. Hence, we applied the identified differentially expressed, phosphorylated proteins to annotated KEGG pathways, and only those pathways were included where at least two proteins were enriched. The KEGG analysis shows a higher number of proteins enriched in metabolic pathways. Of 55 metabolic phosphoproteins, the top two pathways are ribosome and focal adhesion pathways [Figure 3]. The KEGG analysis of identified phosphoproteins shows 55 different molecular pathways enriched with these phosphoproteins [Table 2]. The KEGG PATHWAY MAP shows every differentially expressed phosphorylated protein in the different pathway. (http://www.genome.jp/kegg-bin/show_pathway?13856154838046/ko01100.args).

DISCUSSION
The selective and reversible phosphorylation of proteins is a key regulatory mechanism for biological processes, illustrated by the fact that 30–50% of proteins might
be phosphorylated at any time.[17] Because of low abundance of phosphorylated proteins in cells and tissues, comprehensive analysis of the phosphoproteome is not deeply. The number of phosphorylation site identifications has exponentially increased since the mid-2000s,[18] probably due to the improvement of phosphopeptide enrichment methods such as IMAC[19] or TiO$_2$[20] and antiphospho specific antibody.[21] Among enrichment methods presently available, each has a certain preference or bias within phosphopeptides, so the use of combinations

**Table 2: Different molecular KEGG pathways enriched with phosphor proteins**

| Pathway                                         | Input number | P       |
|-------------------------------------------------|--------------|---------|
| Ribosome                                        | 25           | 2.45E-36|
| Focal adhesion                                  | 10           | 4.40E-08|
| MAPK signaling pathway                          | 7            | 2.99E-04|
| Pathogenic *E. coli* infection - EHEC           | 6            | 2.08E-07|
| Pathogenic *E. coli* infection - EPEC           | 6            | 2.08E-07|
| Alzheimer's disease                             | 6            | 1.77E-04|
| Regulation of actin cytoskeleton                | 6            | 5.37E-04|
| Cell cycle                                      | 5            | 2.31E-04|
| Leukocyte transendothelial migration            | 5            | 2.49E-04|
| Insulin signaling pathway                       | 5            | 4.57E-04|
| Glioma                                          | 4            | 2.33E-04|
| Antigen processing and presentation             | 4            | 7.73E-04|
| Gap junction                                    | 4            | 9.11E-04|
| GnRH signaling pathway                          | 4            | 0.00153231|
| Axon guidance                                   | 4            | 0.03108539|
| CAMs                                            | 4            | 0.003464671|
| Tight junction                                  | 4            | 0.003652718|
| Systemic lupus erythematosus                    | 4            | 0.00447414|
| Calcium signaling pathway                       | 4            | 0.010455247|
| ABC transporters - general                      | 3            | 0.00109524|
| Proteasome                                      | 3            | 0.001411209|
| ALS                                             | 3            | 0.002201735|
| Glycolysis/glucoseoneogenesis                   | 3            | 0.003221659|
| Renal cell carcinoma                            | 3            | 0.004317114|
| Long-term potentiation                          | 3            | 0.005032622|
| Phosphatidylinositol signaling system           | 3            | 0.005221959|
| Adherens junction                               | 3            | 0.005815493|
| ECM - receptor interaction                      | 3            | 0.006891241|
| Small cell lung cancer                          | 3            | 0.007352347|

**Table 2: Contd...**

| Pathway                                         | Input number | P       |
|-------------------------------------------------|--------------|---------|
| ErbB signaling pathway                          | 3            | 0.007589585|
| Apoptosis                                       | 3            | 0.008077528|
| Melanogenesis                                   | 3            | 0.012322205|
| Parkinson's disease                             | 3            | 0.024958773|
| Oxidative phosphorylation                       | 3            | 0.026397894|
| Olfactory transduction                          | 3            | 0.260852689|
| Reductive carboxylic cycle (CO$_2$ fixation)    | 2            | 9.19E-04|
| Nonhomologous end-joining                       | 2            | 0.001835702|
| Glyoxylate and dicarboxylate metabolism         | 2            | 0.002117337|
| Bile acid biosynthesis                          | 2            | 0.007828392|
| C2r3 cycle (TCA cycle)                          | 2            | 0.010059318|
| DNA polymerase                                  | 2            | 0.011895188|
| Nucleotide excision repair                      | 2            | 0.017439469|
| Fatty acid metabolism                           | 2            | 0.018196211|
| Type II diabetes mellitus                       | 2            | 0.018196211|
| mTOR signaling pathway                          | 2            | 0.023868688|
| p53 signaling pathway                           | 2            | 0.040152228|
| Epithelial cell signaling in Helicobacter pylori infection | 2            | 0.041211159|
| B cell receptor signaling pathway               | 2            | 0.046660403|
| VEGF signaling pathway                          | 2            | 0.047780361|
| Colorectal cancer                               | 2            | 0.057082454|
| Toll-like receptor signaling pathway            | 2            | 0.08001462|
| T cell receptor signaling pathway               | 2            | 0.089585578|
| Natural killer cell mediated cytotoxicity       | 2            | 0.132297282|
| Wnt signaling pathway                           | 2            | 0.154290175|

ALS: Amyotrophic lateral sclerosis; CAMs: Cell adhesion molecules; KEGG: Kyoto Encyclopedia of Genes and Genomes; VEGF: Vascular endothelial growth factor; TCA: Tricarboxylic acid; ECM: Extracellular matrix; MAPK: Mitogen-activated protein kinase; *E. coli*: Escherichia coli; EHEC: Enterohemorrhagic *E. coli*; EPEC: Enteropathogenic *E. coli*; mTOR: Mammalian target of rapamycin.
of enrichment methods is a promising approach to achieve high-coverage proteome detection.

Although protein phosphorylation is known to be important for many synaptic processes and in disease, little is known about the global phosphorylation of endometrium tissues. We attempt to evaluate and establish a simplistic protocol to study phosphorylation on endometrium tissues. Here we have presented a large-scale quantitative phosphoproteomics strategy that takes advantage of a TiO$_2$-based phosphopeptide preenrichment step and postfractionation of the isolated phosphopeptides using SIMAC. This setup has allowed for the identification of 2615 phosphopeptides from only 2 mg complex tissue protein per condition. Using TiO$_2$-SIMAC treated samples, 516 proteins that were significantly differentially expressed (fold-change $>$2) in two groups revealed 335 up-regulated and 181 down-regulated phosphorylation sites. These results are more reliable since the MS measurement was carried out with LTQ-Orbitrap Velos mass spectrometer with the mass measurement error $<$5 ppm. The studies carried out with LTQ usually use a 0.8 Da as mass measurement error, which greatly increases the false positive rate.

As shown in Figure 1, further analysis of the whole phosphoproteome in human endometrium tissues indicated...
that phosphorylation on serine (53%) was more common than that on threonine (33%) and tyrosine (14%). The ratio of Ser: Thr: Tyr in our study is 3.8:2.4:1 which was reported in humans is 4:2:5:1. The high ratio of pTyr in our study could be due to TiO₂-based phosphopeptide preenrichment step combined with postfractionation of the isolated phosphopeptides using SIMAC. The prefracionation enrichment setup can also be hampered by the strict optimal peptide-to-TiO₂ ratio, which is much harder to obtain reproducibly. The ratio of Ser: Thr: Tyr in our study is probably because of bias of enrichment method or density of elution acid. It is also likely that the high ratio of pTyr depends on the human endometrium tissues.

GO annotation and analysis of the differences in phosphoproteins of this study were associated with cellular process (ontology: Biological process), cell (ontology: Cellular component), and binding (ontology: Molecular function), which were the dominant processes. Pathway analysis showed 55 different molecular pathways enriched with these phospho proteins and the top two pathways are ribosome and focal adhesion pathways. The focal adhesion pathway has been reported before to be involved in endometriosis development.

Ribosome assembly involves rRNA transcription, modification, folding and cleavage from precursor transcripts, and association of ribosomal proteins. All known ribosomal proteins are associated with either the reversed-phase small (RPS) or reversed-phase large (RPL) subunit. Errors in ribosome biogenesis can result in quantitative or qualitative defects in protein synthesis and consequently lead to the improper execution of the genetic program and the development of specific diseases.

There is evidence that ribosome biogenesis characterize a series of inherited disorders, showing an increased incidence of tumor onset. Specific ribosomal proteins have been showed to have in an extra-ribosomal manner as either tumor promoters (RPL19) or tumor suppressors (RPL5, RPL11, and RPL23 [which is the human homolog for RPL5, RPL11 and RPL17]). Our research indicated that ribosome pathway related genes were enriched at least 2-fold in the genes in human endometrium tissues with endometriosis.

There are 25 ribosome proteins including 10 RPS proteins and 15 RPL proteins in this study, such as RPL19 and RPL23 that have been reported before to be involved in different diseases development. These results demonstrated that interventions in or regulation of this signaling pathway may be some new drugs to therapy for treating endometriosis.

In conclusion, we have successfully utilized LTQ-Orbitrap Velos mass spectrometer to accurately assay the phosphoproteomics of endometrium in women with and without endometriosis. We have identified 516 endometriosis related proteins and analyzed biochemical pathways that are involved in the pathogenesis of this complex disease. Confirmation of these data in a larger and independent patient population, together with identification of all up- and down-regulated proteins and peptides that will increase our understanding of this enigmatic disease. The major weaknesses of the study are the limited number of patients and the lack of depth mechanistic studies. We are collecting more patients to further investigate the correlations between glycoproteome and phosphoproteome of endometrium in women with endometriosis.

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**Conflicts of interest**

There are no conflicts of interest.

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