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Differential Proteomic Profiling of Endometrium and Plasma Indicated the Importance of Hydrolysis in Bovine Endometritis

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INTERPRETIVE SUMMARY

Based on histopathology of uterus, the same type of endometritis specimens were used in iTRAQ analysis. The differentially expressed proteins (DEPs) in uterine tissue and plasma of dairy cows suffering endometritis were demonstrated. The bioinformatic analysis of DEPs discovered that hydrolysis increased at extracellular regions and induced damage to extracellular matrix. It is an important result of a proteomics analysis of cows endometritis. The data of DEPs profiling will be very useful to elucidate the mechanism of endometritis and treatment strategies.

ABSTRACT

Endometritis is an important disease of dairy cows, which leads to significant economic losses in the dairy cattle industry. To investigate alteration of proteins associated with endometritis in the dairy cow, iTRAQ technique was applied to quantitatively identify differentially expressed proteins (**DEPs**) in the endometrium and peripheral plasma from Chinese Holstein cows with endometritis. Compared with the normal (control) group, 159 DEPs in the endometrium and 137 DEPs in the plasma were identified respectively in cows with endometritis. Gene ontology (**GO**) analysis demonstrated that the predominant endometrial DEPs were primarily involved in response to stimulus and stress processes, and mainly played a role in hydrolysis at the

extracellular region. The predominant plasma DEPs were mainly components of the cytosol and non-membrane-bounded organelles, and were involved in response to stress and regulation of enzyme activity. Protein-protein interaction of tissue DEPs revealed that some core seed proteins such as RAC2, ITGB2 and CDH1 in the same network with CD14, MMP3 and MMP9, had important functions in cross talking of pathways related to extracellular proteolysis. In summary, it is proposed that significant enzymatic hydrolase activity in the extracellular region may be a molecular mechanism through which the altered proteins may promote inflammation and hence endometritis.

Key words: iTRAQ; bovine endometritis; differentially expressed proteins; hydrolysis

INTRODUCTION

Uterine infection and endometritis are important causes of infertility and reproductive losses in dairy cattle worldwide (Gilbert et al., 2012; LeBlanc et al., 2014). The protective physical barriers to contamination provided by the uterine luminal epithelium, cervix, vagina and vulva are all disrupted during normal parturition (Sheldon and Dobson, 2004). These, in addition to the large volumes of fluid and tissue debris in the uterine lumen, both allow bacteria from the environment or the animal's faeces to contaminate the uterus postpartum (Bondurant, 1999; Sheldon and Dobson, 2004). Observations from several studies showed the presence of bacteria in the uterus of more than 90 % of cows within the first two weeks after calving (Dohmen et al., 1995; Sheldon et al., 2008). Although most of the bacterial contaminants are gradually cleared from the uterus via uterine involution and innate immune response (Azawi, 2008; Singh et al., 2008), up to 20 % of cows have clinical metritis, while 20 % to over 50 % of cows subsequently develop subclinical inflammation of the uterus (endometritis) beyond 3 weeks postpartum (Sheldon et al., 2009a; LeBlanc, 2014). Endometritis cause uterine tissue damage, embryonic death and early abortion, delayed onset of ovarian cyclicity, extended luteal phases, and reduced conception rates in affected cattle (Sheldon et al., 2009b; Gilbert, 2012). These problems lead to infertility and huge financial losses in the dairy industry (Sheldon et al., 2009a).

The endometrium has crucial roles in female reproduction including the regulation of the oestrous cycle, sperm transit, nourishment of early embryo and formation of the placenta. Furthermore, the endometrial luminal epithelial cells and the underlying stromal cells express pathogen recognition receptors and mount innate immune response to microbes or microbial ligands (Hickey et al., 2011; Oguejiofor et al., 2015a;

Oguejiofor et al., 2015b). The major bacteria isolated from cows with endometritis were *Escherichia coli* and *Trueperella pyogenes*, followed by other anaerobic species, such as *Fusobacterium* and *Bacteroides* (Dohmen et al., 2000; Williams et al., 2005; Bicalho et al., 2012). Although the effects of different types of bacteria on uterine disease mechanisms is not completely understood (Westermann et al., 2010), it is known that unregulated inflammation can lead to disease (Maybin et al., 2011). This can impair reproductive performance not only during the infection but also after resolution of clinical signs of endometritis (Plontzke et al., 2010; LeBlanc, 2014). Interestingly, there is evidence that certain viruses such as bovine viral diarrhea virus (Grooms, 2004; Oguejiofor et al., 2015a; Cheng et al., 2016) and bovine herpes virus (BoHV)-4 (Donofrio et al., 2009) have important aetiological contribution to the emergence of uterine disease.

Endometritis is described histopathologically as a superficial inflammation of the endometrium, with histological evidence of inflammation (Sheldon et al., 2006). Endometritis has been defined based on the determination of abnormally increased polymorphonuclear cells (PMN) in uterine luminal fluid (Kasimanickam et al., 2004; Gilbert et al., 2005) or endometrial biopsy tissue (Galvão et al., 2011). In addition, there was increased endometrial expression of inflammatory genes including granulocyte chemotactic protein 2 (CXCL5), interleukin (IL-1β and IL-8), tumor necrosis factor (TNF) (Fischer et al., 2010; Kasimanickam et al., 2014). Also, elevated levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10) in serum were used to indicate the development of bovine endometritis (Islam et al., 2013; Kasimanickam et al., 2013). Previously, genome-wide transcriptomic profiling of bovine endometrium using mRNA-Seq showed that immune activation and inflammation preceded tissue proliferation and repair in the healthy postpartum endometrium (Foley et al., 2012). This highlighted the importance of a balanced inflammatory immune response as key to sufficient bacterial clearance and restoration of an endometrial environment capable of supporting a new pregnancy (Jabbour et al., 2009). There are limited studies on the bovine uterine proteome. One study identified 9 proteins using two-dimensional electrophoresis (2-DE) to compare the proteins present in the uterine luminal fluid between pregnant and non-pregnant animals (Ledgard et al., 2009). Another used two-dimensional fluorescence difference in-gel electrophoresis (2D-DIGE) to compare the proteome of the

pregnant and non-pregnant endometrium (Berendt et al., 2005). However, there is little available information on the proteomic profile of cows with endometritis. A previous study identified 14 endometritis-associated proteins using two-dimensional electrophoresis (Choe et al., 2010).

Recent developments in the field of proteomics have led to a renewed interest in animal disease diagnosis and treatment. The isobaric tags for relative and absolute quantification (**iTRAQ**) is the most popular technique, which combined with multidimensional liquid chromatography (LC) and tandem MS, is used to study differentially expressed proteins. The aim of this study is to use the iTRAQ technique to characterize the proteomic changes in endometrial tissue and plasma from Chinese Holstein dairy cows with endometritis. The bioinformatics analysis of differential proteome may create new understanding on the main proteins or pathways associated with bovine endometritis, and form the basis for future diagnosis and treatment of diseased animals.

MATERIALS AND METHODS

Animals and Samples Collection

Sample collection was carried out under license in accordance with national guidelines (Ministry of Agricultural of China 2015/No.18). Rectal palpation and cervico-vaginal mucus observation were conducted to identify normal animals or cows with endometritis. Endometrial biopsies and blood specimen were collected from Chinese Holstein cows, aged from 2 to 5, 21 - 35 days postpartum. Ten candidate cows had obvious signs of clinical endometritis (i.e. presence of purulent or mucopurulent vaginal discharge), but apparently did not show any symptoms of other local or systemic diseases except with respect to the focus of this study in the reproductive tract. Other 10 cows without signs of endometritis were categorized as the normal control animals. Briefly, 5 ml blood was took from the jugular vein, then collected into commercial vacuum tubes and mixed properly with the anticoagulant $EDTAK_2$. This was centrifuged at 3, 000 rpm for 15 min, and 0.5 mL of plasma was recovered and quickly preserved in liquid nitrogen. Endometrial biopsies were quickly frozen in liquid nitrogen and were fixed in 4 % formalin respectively. After formalin fixation, tissue specimens was embedded in paraffin blocks using routine procedures, followed by haematoxylin and eosin (HE) staining and histopathological examination under the microscope to identify pathological state.

Protein Isolation, Digestion and Labeling with iTRAQ Reagent

Protein extraction was performed on 4 non-endometritis samples and 4 endometritis samples, which were selected according to histopathological results. Briefly, the frozen tissue was ground into powder and then dissolved in Lysis buffer Ⅰ (7 M urea, 2 M thiourea, 4 % propanesulfonic acid, 40 mM Tris-HCl, pH 8.5) containing complete protease inhibitor. The cells were lysed by sonication at 200 W for 15 min and then centrifuged at 4 °C, 30, 000 g for 15 min. The supernatant was mixed with 5× volume of chilled acetone containing 10 % (vol/vol) trichloroacetic acid, incubated at -20 °C overnight, and then centrifuged as described. The precipitate was washed three times in chilled acetone. The recovered pellet was air-dried and dissolved in Lysis buffer Ⅱ (7 M urea, 2 M thiourea, 4 % Nonidet P40, 20 mM Tris-HCl, pH 8.0-8.5). The suspension was sonicated, centrifuged as described, and transferred to another tube. The disulfide bonds were reduced by treatment with 10 mM Dithiothreitol, whereas cysteines were blocked by treatment with 55 mM Iodoacetamide. Then the supernatant was washed in acetone and centrifuged as described. Recovered pellets were air-dried, dissolved in 500 μL 0.5 M TEAB (Applied Biosystems, Milan, Italy), and then sonicated one more time. Finally, samples were centrifuged, and supernatant was transferred to a new tube and quantified using Bradford method. The proteins in the supernatant were kept at -80 °C for further analysis. Correspondingly, the plasma samples (matching the endometrial tissues) were cleared of high-abundant proteins using ProteoMiner kit (Bio-rad, USA), and other processes, including washing, precipitation, centrifugation, sonication, and the reduction of disulfied bonds, were performed as already described.

Total protein (100 μg) was measured from each sample solution and the protein was digested at 37 °C for 16 h with Trypsin Gold (Promega, Madison, WI, USA) with the ratio of protein to trypsin of 30:1. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacture's protocol for 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 μL isopropanol. Samples were labeled respectively with different isobaric tags, and incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

Liquid Chromatography–Electrospray Ionization Tandem Mass

Spectrometry (LC-ESI-MS/MS) Analysis Based on Q-exactive

The iTRAQ labeled peptide mixtures were fractionated by strong cation exchange chromatography,

which was conducted on a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) using a 4.6 × 250 mm Ultremex SCX column containing 5 μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffers as follows: buffer A (25 mM NaH_2PO_4 in 25 % ACN, pH 2.7) for 10 min, 5-60 % buffer B (25 mM NaH_2PO_4, 1 M KCl in 25 % ACN, pH 2.7) for 27 min, and 60-100 % buffer B for 1 min. The system was then maintained at 100 % buffer B for 1 min before equilibrating with buffer A for 10 min prior to the next injection. Under a monitoring absorbance of 214 nm, a total of 20 fractions were collected, then desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

Each fraction was dissolved in buffer liquid (2 % ACN, 0.1 % FA) and centrifuged at 20, 000 g for 10 min, the final concentration of peptide was about 0.5 μg/μL on average. 10 μL supernatant was loaded on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column (inner diameter 75 μm). The peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in an QEXACTIVE (Thermo Fisher Scientific, San Jose, CA) coupled online to the HPLC. Intact peptides were detected in the qExactive orbitrap at a resolution of 70, 000. Peptides were selected for MS/MS using high-energy collision dissociation operating mode with a normalized collision energy setting of 27.0; ion fragments were detected in the Orbitrap at a resolution of 17500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the 15 most abundant precursor ions above a threshold ion count of 20, 000 in the MS survey scan with a following Dynamic Exclusion duration of 15 s. The electrospray voltage applied was 1.6 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the orbitrap. The AGC target for full MS and MS2 was 3E6 ion counts and 1E5 ion counts, respectively. The m/z scan range was 350 to 2, 000 Da. For MS2 scans, the m/z scan range was 100-1, 800 Da.

Data Analysis

MS raw data were converted into Mascot generic file (MGF) using Proteome Discoverer 1.2 (PD 1.2, Thermo), and the MGF data file were searched by using Mascot search engine (Matrix Science, London, UK; version 2.3.02) to identify proteins. Each confident protein identification involves at least one unique peptide. For protein quantification, it was required that a protein contains at least two unique spectra. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot (http://www.matrixscience.com/help/quant_statistics_help.html). We only used ratios with *P*-values < 0.05,

and only fold changes of > 1.5 or <0.67 for endometrial tissue and > 1.2 or <0.67 for plasma were set as the criteria to determine up or down-regulated proteins, respectively.

Bioinformatics Analyses

Functional annotations of proteins were performed using Blast2GO program against the non-redundant protein database (NR, NCBI). Gene Ontology (GO) is an international standardization of gene function classification system. It has 3 Ontologies which can describe molecular function, cellular component, and biological processes respectively. The main types of annotations were obtained from the gene ontology consortium website (http://david.abcc.ncifcrf.gov/). Venn diagram was drawn using online venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The protein-protein interaction network was analyzed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software (http://string.embl-heidelberg.de/). The differentially expressed proteins were compared by a hierarchial cluster analysis using Cluster 3.0. The data were displayed using Tree View software.

Western Blot

The same endometrial tissues that were used for iTRAQ analysis were divided into non-endometritis group and endometritis group. Total proteins were extracted from each sample using a commercial kit (BestBiotech, Shanghai, China). Protein concentration was determined using the BCA Protein Assay Kit (Biomiga, Shanghai, China). Briefly, equivalent amounts of protein (20 μg) were loaded on 12 % SDS-PAGE gels and then transferred onto 0.45 μm nitrocellulose membrane (Millipore Corporation, Billerica, USA). After blotting, the membrane was blocked with 5 % skimmed milk and incubated with primary antibody and second antibody at 37 °C for 60 min, respectively. The incubated membrane was then washed in 0.05 % PBST three times. Detection was performed using chemiluminescence luminol reagents (CWbiotech, Beijing, China) and recorded by film exposure. The polyclonal antibodies to serum amyloid A (sc-20275), myeloperoxidase (sc-16128-R), matrix metalloproteinase 3 (sc-6839), fibrinogen (sc-33916), integrin β6 (sc-6632), and integrin β2 (sc-6623) were purchased from Santa Cruz Biotechnology (California, USA). Mouse monoclonal antibody to beta actin (ab8226) and goat polyclonal antibody to lactoferrin (ab112968) were obtained from Abcam (Cambridge, UK). The horseradish peroxidase (HRP) conjugated goat anti-mouse IgG and rabbit anti-goat IgG were obtained from Proteintech (Wuhan, China).

RESULTS

Histopathology of Endometrial Tissue

A representative section showing typical histopathologic changes observed in the endometrial tissue specimens from cows with endometritis are shown in Figure 1. The normal endometrium comprises a single layer of columnar luminal epithelial cells. The endometrial gland lies deeper in the stromal layer and comprises the glandular epithelia which release their secretions into the lumen of the gland. There was little presence of red blood cells and migrant granulocytes and lymphocytes in the lamina propria (Figure 1A). During inflammation in the uterus or endometritis (Figure 1B), there was degenerative necrosis of the luminal epithelial cells with nuclear condensation and damage of the mucous layer and basement membrane. There was expansion of the capillaries in the lamina propria, with significant infiltration of red blood cells, and leukocytes, resulting in a general increase in cell density. This was typical of hemorrhagic endometritis (Figure 1B).

Protein Profile by iTRAQ-coupled 2D LC-MS/MS Analysis

A total of 3493 proteins were determined by iTRAQ-coupled 2D LC-MS/MS analysis in the endometrial tissue. Among them, 159 proteins displayed significantly altered levels with fold cut-off of ≧ 1.5. As shown in Supplementary table 1, these significantly altered proteins include 109 increased and 50 decreased ones. Analysis of the plasma identified 137 DEPs with fold cut-off of ≧ 1.2, out of which 49 proteins were increased while 88 were decreased (Supplementary table 1).

Functional Classifications of Differentially Expressed Proteins

Functional annotation of the DEPs was initially performed using Blast2GO program. Three main types of annotations were obtained from the gene ontology consortium website:cellular component, metabolic functions, and biological processes. 159 proteins were significantly altered in endometritis uterus, and enrichment analysis of biological processes showed that endometritis primarily affected response to stimulus and stress (Figure 2A). Enrichment analysis also identified hydrolase activity as a predominant event for stress and stimulus during inflammation (Figure 2C). Furthermore, cellular component-based enrichment analysis identified the significantly altered proteins to be located at the extracellular region (Figure 2B). The same GO analysis was conducted on 137 proteins identified in the plasma. Biological processes identified DEPs mainly involved in stress response (Figure 3A), and molecular function regulation of enzymes activities (Figure 3C). It was observed that some plasma DEPs were involved in components of non-membrane-bounded organelle

and cytoskeletal processes (Figure 2B), such as PDLI2, COR1A, ANXA1, and RL14 (Supplementary table 2).

Protein Distribution Between Endometrium and Plasma

There were 159 proteins differentially expressed in the endometrial tissue, and 137 proteins in the plasma. However, only 9 proteins were identified to occur in both endometrial tissue and plasma. Of these proteins, 3 were similarly increased and 2 were similarly decreased in the two specimens; 3 proteins were increased in the tissue, but decreased in the plasma; 1 was increased in the plasma, but decreased in the tissue. The distribution of these proteins is shown in a Venn diagram (Figure 4). Cluster analysis was performed for the 9 proteins as shown in the dendrogram (Figure 4). These include hemoglobin subunit beta (HBB), resistin (RETN), serum amyloid A protein (SAA), inter-α-trypsin inhibitor heavy chain H2 precursor (ITIH2), IgA heavy chain constant region (IGHA1), immunoglobulin gamma 1 heavy chain constant region (IGHGa), CD14, matrix metalloproteinase 3 (MMP3), and serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10 (SERPINA10).

Validation of Changes in Protein Level by Western Blot

In order to validate the differentially expressed proteins identified by the iTRAQ analysis, seven increased proteins were selected for Western blot analysis, including Serum amyloid A (SAA), lactoferrin (LTF), myeloperoxidase (MPO), matrix metalloproteinase 3 (MMP3), fibrinogen (FG), integrin β6 (IGB6), and integrin β2 (IGB2). Equal amounts of proteins from endometrial tissue were detected with antibodies as shown in Figure 5. The results showed that the ratios of the selective proteins were consistent with those obtained from the iTRAQ analysis.

Analysis of Protein-Protein Interaction

The interactions between proteins are complex and interrelated during disease development. Studying the interaction network is of strategic importance to researchers to understand how disease-related proteins interact with each other and how they affect cell function. To investigate these issues, STRING database was searched for protein interactions to the 159 DEPs in endometrial tissue (Figure 6). As observed from the network, RAC2, ITGB2, and CDH1 were the core seed proteins in the interaction network, and they had important functions in several signal transduction pathways including MAPK signaling pathway, VEGF signaling pathway, Wnt signaling pathway, focal adhesion, adherens junction, cell adhesion molecules

(CAMs), leukocyte transendothelial migration, and regulation of actin cytoskeleton.

DISCUSSION

Endometritis is prevalent in dairy herds worldwide causing significant economic losses resulting from infertility (Sheldon et al., 2009a). Nevertheless, research on endometritis in dairy cows is limited by high costs, a long gestation period, and lack of uniform genetic backgrounds. With the development of omics research, proteomics is emerging as a useful tool in the study of endometritis in cows. Understanding the molecular mechanisms of endometritis at the protein level is likely to contribute to the design of new methods in disease control and fertility enhancement.

Diagnosis of endometritis in cows is based on the presence of pathologic vaginal discharge. In the past 10 years, a standardized definition and characterization of uterine disease was suggested by several authors (Williams et al., 2005; Barlund et al., 2008; LeBlanc et al., 2011). People well known that different physiological condition may influence cow's endometrium including DIM, estrus cycle, and age. However, it is difficult to correct for all variables when working with farm animals such as cattle as opposed to controlled laboratory animals and conditions. We were unable to determine the exact days of the oestrous cycle and DIM of the animals, and therefore cannot account for any influence due to ovarian hormones. In the absence of the accurate information of cow's physiology, we herein conducted histopathologic study using HE staining to characterize endometritis (Figure 1), and only tissues with the same type of hemorrhagic endometritis were selected for proteomic analysis.

According to the results of iTRAQ analysis, approximately 3500 bovine proteins were identified, and therefore using a *P*-value cut-off of < 0.05, 159 and 137 proteins were found to expressed differentially in the endometrial tissue and plasma respectively (Supplementary table 1, 2). As shown in Supplementary table 1, DEPs in endometrial tissue of cows with endometritis included many specific and non-specific immunity-associated proteins, providing evidence of on-going inflammatory activity. Five cathelicidin proteins (Cathelicidin-2, -3, -5, -6, -7), a family of host-defence peptides, were significantly increased. These are small amphipathic peptides that exhibit rapid and potent activity against many pathogens, including bacteria, viruses, and fungi (Braff et al., 2005).

The complement proteins, complement component 1, r subcomponent (C1R) and complement component 1, s subcomponent (C1S) were also increased in the endometrium. Complement proteins

are important in innate and adaptive immunity by opsonisation of pathogens, recruitment of phagocytes to the site of infection, and direct killing of pathogens through the formation of the membrane-attack complex (Medzhitov, 2007). Here, several complement proteins were also increased in the plasma of cows with endometritis including complement component-7 (C7) and -9 (C9), complement factor-H (CFH), -D (CFD), -properdin (CFP) and complement C4-A Precursor (C4A13). The presence and activity of serum complement proteins in the bovine uterus during endometritis in not well known. However, physiological haemorrhage from the endometrium and the increased vascular permeability due to mucosal inflammation, are likely to facilitate the leakage of cellular and serum components, including complement, to the uterine lumen (Singh et al., 2008).

The protein S100A12 is a member of the S100A calcium-binding proteins that are known to possess inflammatory and innate immune properties against different pathogens (Foell et al., 2013; Zackular et al., 2015). Antimicrobial peptides (AMPs), including defensins, have innate and adaptive immune properties and are secreted at the mucosal surface of the female reproductive tract (Hickey et al., 2011; Oppenheim et al., 2003). We observed increased protein levels of S100A12 and beta-defensin (BDFN) in the endometrial tissue during endometritis which reflects previous marked expression of *S100* genes and several AMP genes in cows with severe endometrial inflammation (Wathes et al., 2009). We also observed increased levels of LTF protein which is known to have important roles in chemotaxis, activation of immune cells, antigen processing, and adaptive immune response (Puddu et al., 2009).

Acute phase proteins (APP) are blood proteins primarily synthesized by hepatocytes as part of the acute phase response (APR), which is part of the early-defense or innate immune response to different stimuli including infection and inflammation (Cray et al., 2009). Here, we observed increased levels of the APP, Serum amyloid A protein (SAA) and Haptoglobin (HP) in the tissues with endometritis (Supplementary table 1). Interestingly, these proteins were not significantly elevated in the plasma of the same cows with endometritis. Moreover, SAA-1, -4 and C-reactive protein (CRP) showed a tendency for decreased levels in the plasma of these cows. Previously, the plasma concentrations of the APP, α1-acid glycoprotein (AGP), ceruloplasmin and HP were increased in postpartum cows with uterine bacterial contamination but these returned to basal levels within 14-21 days of uterine involution (Sheldon et al., 2001). Although plasma concentration of HP and SAA were higher in cows with postpartum metritis (Huzzey et al., 2009; Chan et al.,

2010), the levels of these proteins in prolonged uterine disease (subclinical or clinical endometritis) are largely unknown. The gene transcripts for APPs (*HP* and *SAA3*) were increased in the endometrium or endometrial cells in response to bacterial presence or inflammation (Gabler et al., 2010; Chapwanya et al., 2012) but not in cows with subclinical or clinical endometritis (Fischer et al., 2010). However, it is not clear if APPs are derived entirely from the liver or if the endometrial cells can secrete significant levels of these proteins locally in response to infection or inflammation, since our findings suggest the latter. On the other hand, endometrial cells infected with *E. coli* or exposed to bacterial LPS increased their gene expression of *HP* and *SAA3* (Chapwanya et al., 2013; Oguejiofor et al., 2015b) but the secreted protein concentrations of HP and SAA were not altered (Davies et al., 2008).

Furthermore, we did not observe marked changes in the concentrations of inflammatory cytokines both in the endometrial tissue and plasma from cows with endometritis. The expression of these cytokines is known to differ depending on the time postpartum and the health condition of the uterus, but their roles in the development of endometritis is not clear. Several previous studies associated the differences in endometrial mRNA expression of inflammatory cytokines e.g. *IL1*, *IL6*, *IL8* and *TNF* with the development of clinical or subclinical endometritis (Gabler et al., 2009; Fischer et al., 2010; Kasimanickam et al., 2014). The protein concentration of inflammatory cytokines in the endometrial tissue during endometritis is not known. Their concentrations in uterine flush were observed to differ in cows with clinical or subclinical endometritis (Kim et al., 2014). Moreover, the serum concentration of proinflammatory cytokines were increased in the cows with clinical and subclinical endometritis in one study (Kasimanickam et al., 2013) but there were no differences in another study (Kim et al., 2014).

The results of GO enrichment analysis of endometrial DEPs showed that the most predominant proteins were proteolytic enzymes distributed at the extracellular region, which were activated to respond to stress and stimulus during endometritis (Figure 2). Matrix metalloproteinases (MMPs) are a family of mainly extracellular zinc-dependent endoproteinases whose prominent functions include the proteolytic degradation of the extracellular matrix (ECM) proteins and tissue membranes as well as the remodelling of biologically active proteins during morphogenesis, tissue development, wound healing, and reproduction (Page-McCaw et al., 2007; Klein and Bischoff, 2011). MMPs also regulate inflammation and innate immunity by modulating cytokine and chemokine activity in recruiting leucocytes to the site of inflammation (Parks et al., 2004; Van

Lint and Libert, 2007). Uterine infection may alter the levels of MMPs and therefore interfere with the mechanism of endometrial repair in the postpartum uterus. Bovine endometrial cells respond to bacterial LPS by increased gene expression of *MMP1*, *MMP3* and *MMP13* (Oguejiofor et al., 2015b). The increased endometrial expression of these MMPs, in addition to *MMP9*, was also associated with delayed uterine remodelling in postpartum cows (Wathes et al., 2011). We observed significant increase in both MMP3 and MMP9 proteins in the endometrial tissue during endometritis (Supplementary table 1, Figure 5). MMP9 is a gelatinase that can digest a number of other ECM molecules while MMP3 is a stromelysin that can also activate other MMPs (Nagase et al., 2006). We hold that increased MMPs promoted inflammation, and played a role in the ECM degradation and cell/basement membrane damage in endometrial tissue during endometritis (Figure 1B). It is known that an imbalance in the proteolytic and anti-proteolytic activity of MMPs could lead to several conditions ranging from tissue destruction in chronic inflammation to cancer metastasis (Klein and Bischoff, 2011). Although ECM remodeling is critical to endometrial receptivity for the embryo (Bauersachs et al., 2006), escalated MMPs proteolytic activities are likely to cause chronic inflammation and a hostile uterine environment for reproduction.

In addition to MMPs, many other proteins are also known to have extracellular proteolytic activities. Moreover, proteins rarely carry out their functions alone, but often by physically interacting with other proteins (Spirin and Mirny, 2003). In this study, protein-protein interaction (PPI) was analyzed using STRING to reveal functional links among the endometrial tissue proteins that were significantly altered in endometritis (Figure 6). The PPI data are commonly shown as networks, which include a node (a protein) and an edge (interaction between proteins). The PPI data are used extensively to assign or predict protein function (Xiong et al., 2013; Yu et al., 2015) and to provide more insight into molecular mechanisms of biological processes (Huang et al., 2015). According to neighborhood counting approach of protein function prediction, interacting proteins may have some similar functions (Chua et al., 2006; Ng et al., 2010). Our results identified significant enzymatic hydrolase activity in the extracellular region as a possible molecular mechanism through which the differentially expressed proteins may promote inflammation and endometritis. Therefore, some core seed proteins such as RAC2, ITGB2 and CDH1 in the same network with CD14, MMP3 and MMP9 may be related to extracellular hydrolase activity and proteolysis (Figure 6).The definite roles of the identified core seed proteins RAC2, ITGB2, and CDH1 in this hydrolase activity is not clear, but these proteins have critical roles

in cell signalling, migration and adhesion (Ridley, 2006; Sarantos et al., 2008; West and Harris, 2016). Their interactions with CD14, a co-receptor involved in bacterial recognition and innate immune response (Kumar et al., 2011), and MMPs highlights the complex network of recognition molecules, adhesion molecules, proteolytic factors and migration of immune cells in inflammatory process and endometritis. Essentially, the functional roles of adhesion molecules in cell proliferation (George and Dwivedi, 2004) and leucocyte migration (Madri and Graesser, 2000) are known to be facilitated by the cleavage and proteinase activity of MMPs. Therefore, the protein network complemented GO annotation, and provided some detailed information about the interactions of DEPs in endometritis. Some of these important seed proteins were determined by Western blot which validated the fold changes from iTRAQ analysis in endometrial tissue (Figure 5).

Additionally, differentially altered proteins in the plasma were mainly components of the cytosol and non-membrane-bounded organelles involved in response to stress and regulation of enzyme activity (Figure 3). A recent study showed that bovine uterine proteome is dynamic, with the concentrations proteins including enzymes, antioxidants and immune molecules in the uterine fluid differing from that of plasma (Faulkner et al., 2012). Interestingly, there were 9 overlapped DEPs (Figure 4) in the endometrial tissue and plasma. Endometrial proteome may have comprised of some blood-derived proteins. Thus, it is possible that some increased proteins were transferred from blood to the uterus such as HBB, SAA, and RETN (Figure 4). However, many plasma proteins also differed significantly from the endometrium (Supplementary table 2, Figure 4). Although plasma and endometrial tissue came from the same cows, it was not confirmed if blood cell damage was from the inflammatory lesion of the endometrial tissue. It was observed that the predominant DEPs in endometrial tissue were extracellular (Figure 2), whereas the main plasma DEPs were intracellular (Figure 3). This indicated that cell damage may have also taken place in the peripheral vessels instead of uterine endometrium that is actively regulated by local factors. After all, the blood system is sensitive to any other disease or dysfunction. Therefore, endometrial proteome profiling is thought to be more significant to aid our understanding of endometritis mechanism as well as contribute to the exploration of disease treatment.

CONCLUSION

We used the iTRAQ proteomic analysis to determine proteins alteration in endometrial tissue and plasma of Chinese Holstein dairy cows with endometritis. It identified 159 and 137 differentially expressed proteins in endometrial tissue and plasma respectively. Profiling of the endometrial DEPs demonstrated that host

defense was activated to protect the endometrium from infection and inflammation. However, bioinformatics analysis of DEPs indicated that extracellular hydrolase activity was significantly increased in uterine tissue. Therefore, we propose that inflammatory damage induced by hydrolysis may contribute significantly to the aggravation of endometritis. These findings can facilitate further studies to better understand the molecular mechanisms through which altered proteins may promote inflammation and hence endometritis.

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Fig. 1

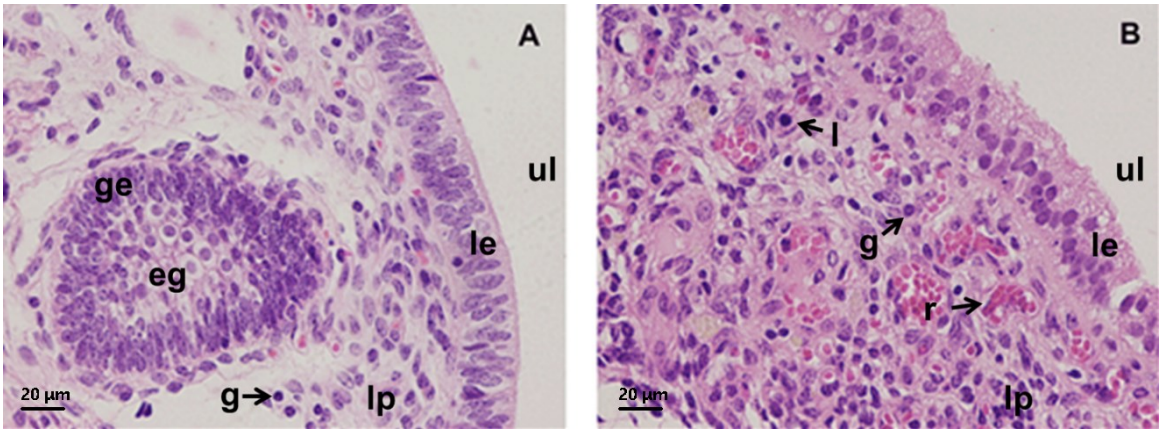


Figure 1. Histopathologic change in endometrial tissue without endometritis (A) and with endometritis (B) investigated by HE stain (400×). Micrographs of the bovine endometrium showing normal tissue (A) and histopathologic changes observed during endometritis (B). Uterine lumen (ul), endometrial gland (eg), luminal epithelium (le), glandular epithelium (ge), lamina propria (lp), granulocytes (g), lymphocytes (l), red blood cells (r).

Fig.2

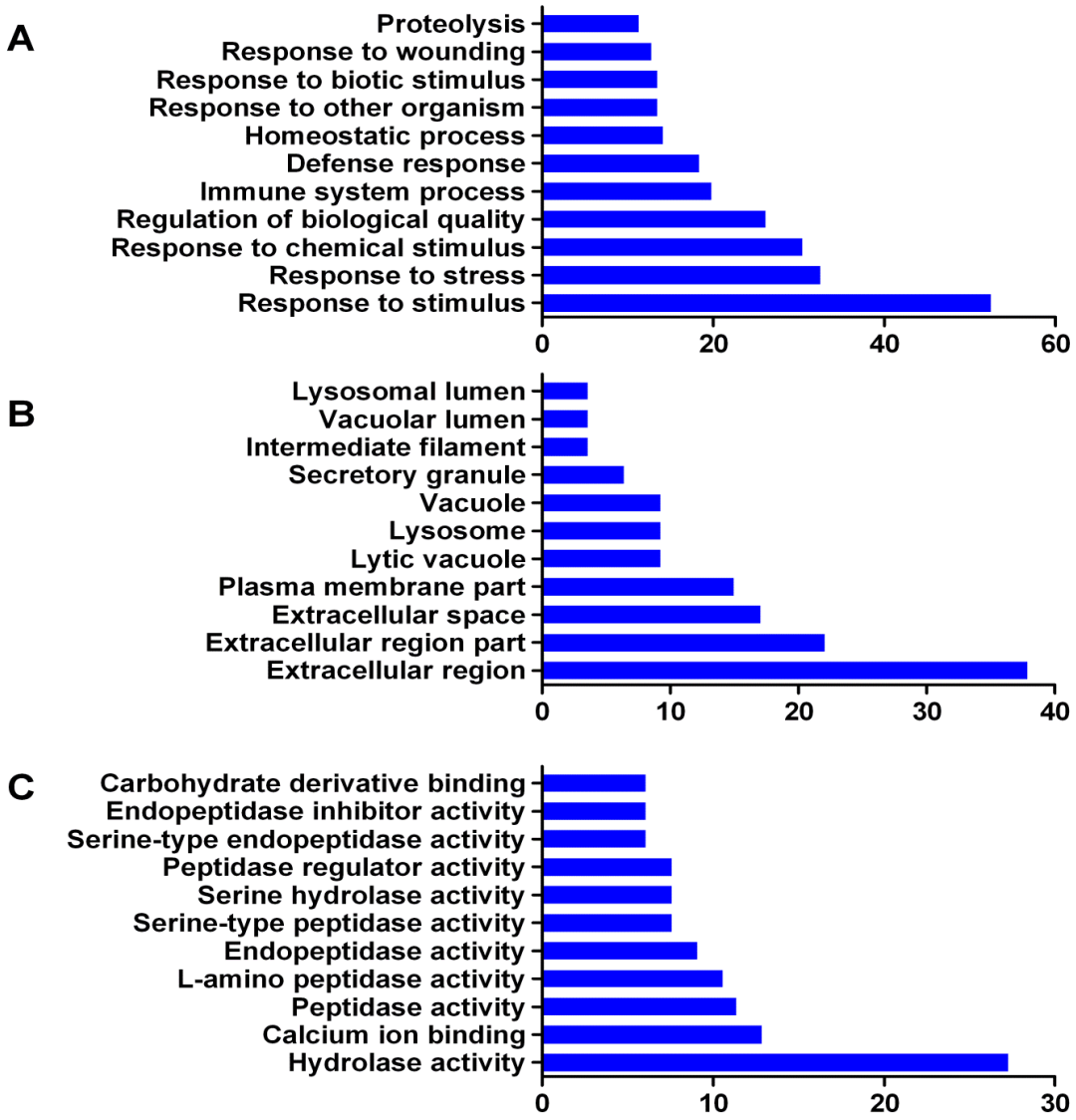


Figure 2. Top 11 classification of identified proteins differentially expressed in the endometrium based on their functional annotations using the Gene Ontology enrichment analysis. (A) biological processes; (B) cellular components; (C) molecular functions.

Fig. 3

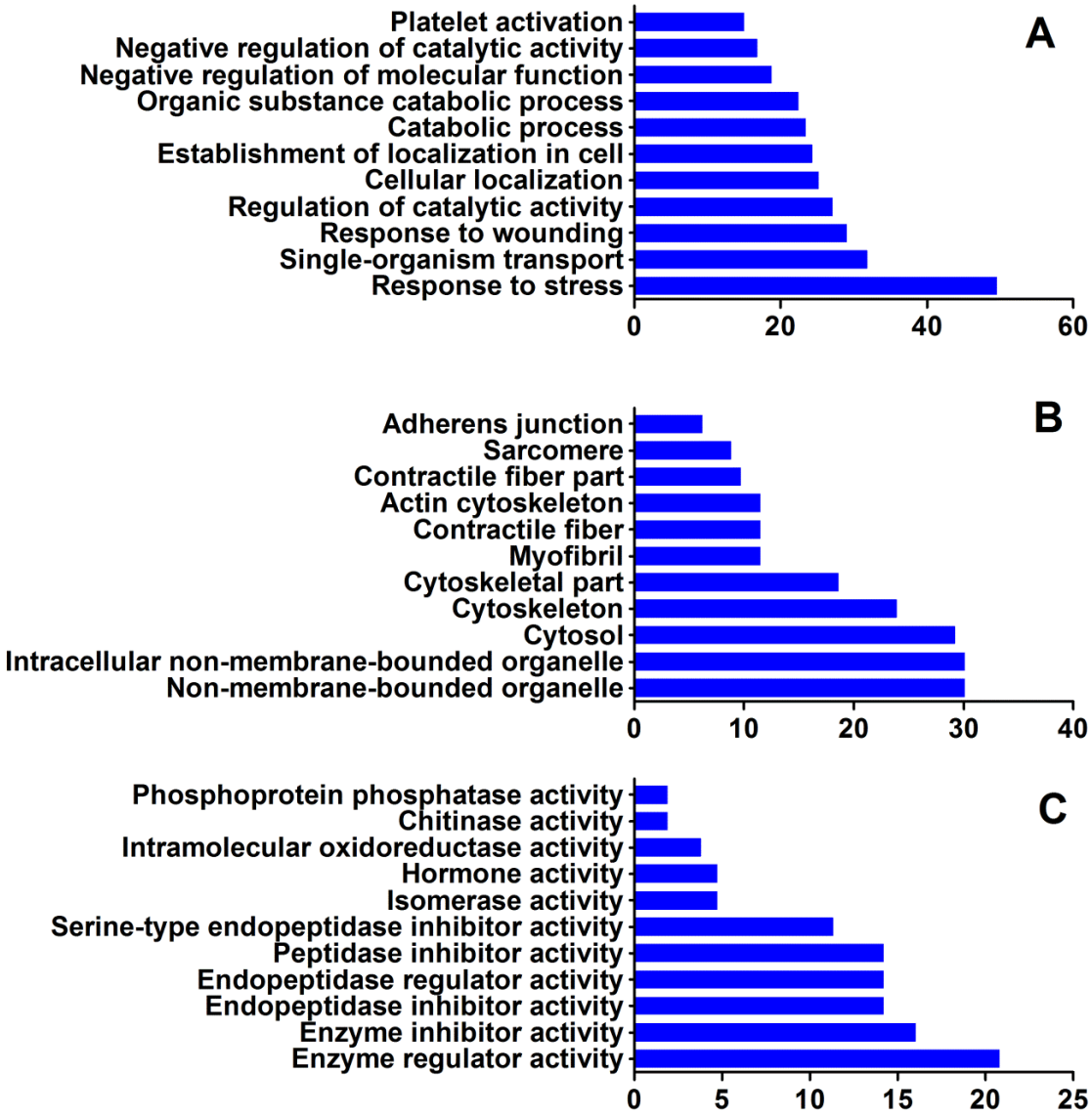
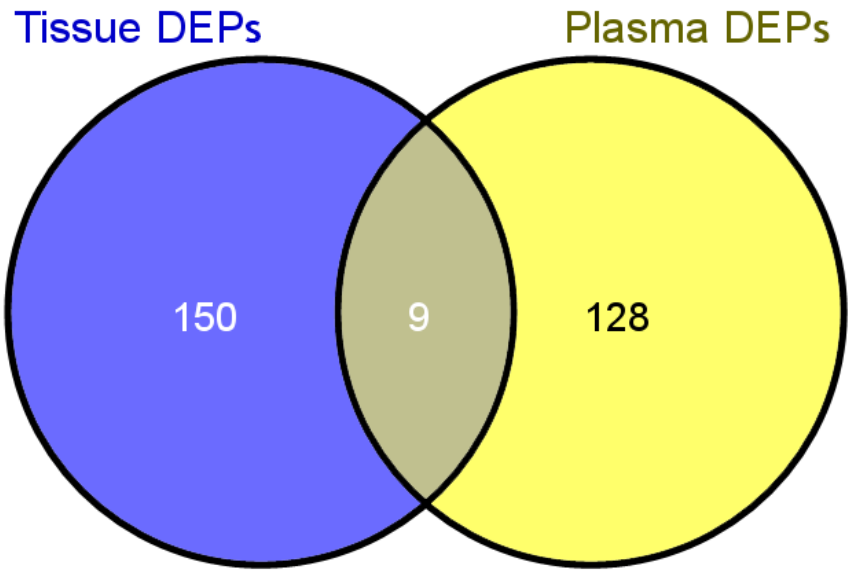


Figure 3. Top 11 classification of the identified proteins differentially occurring in plasma based on their functional annotations using the Gene Ontology enrichment analysis. (A) biological processes; (B) cellular components; (C) molecular functions.

Fig. 4

A



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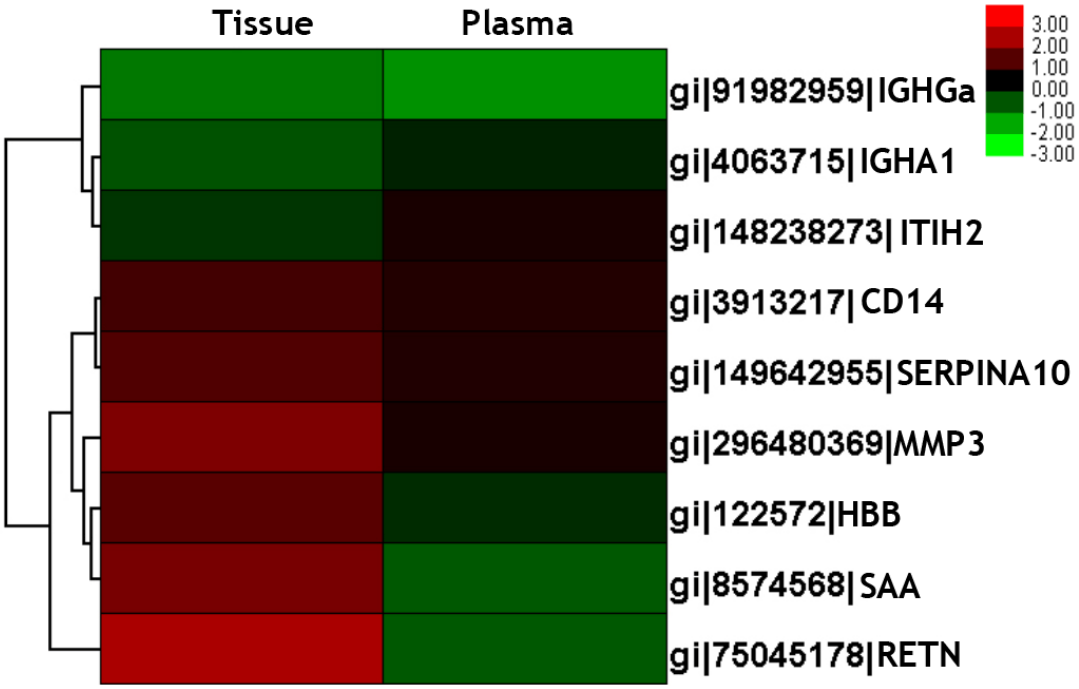

Figure 4. Venn diagram showing the distribution of proteins between endometrial tissue and plasma (A), and cluster analysis of 9 shared DEPs in tissue and plasma (B).

Fig. 5

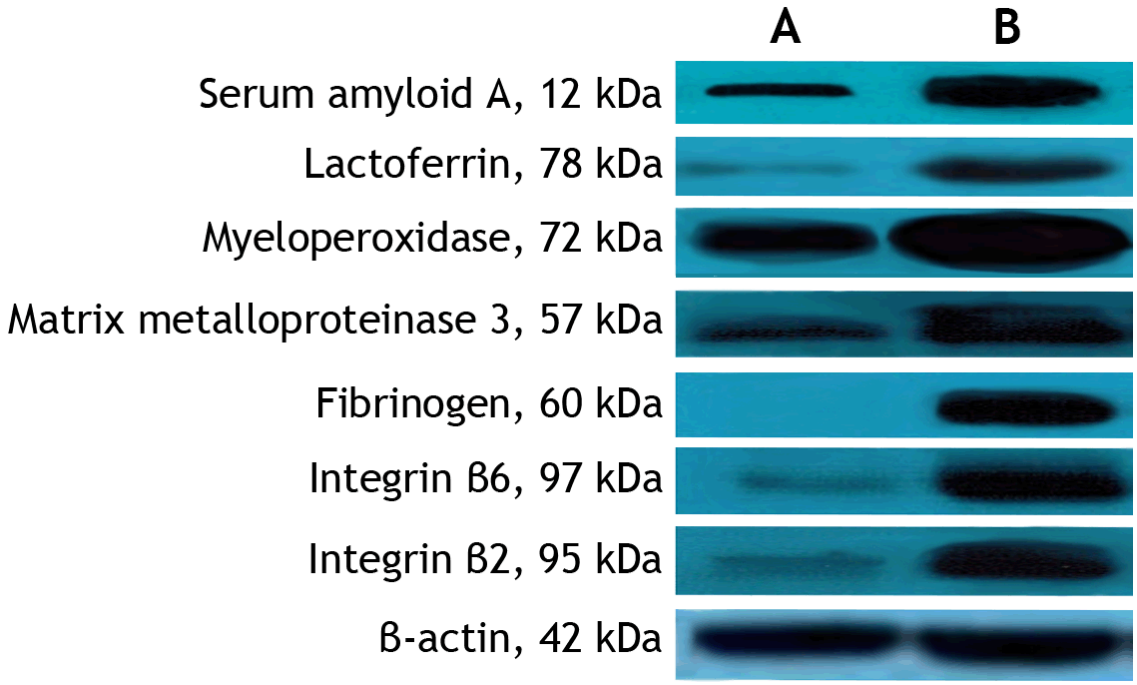

Figure 5. Confirmation of seven differentially expressed proteins by Western blot analysis. (A) non-endometritis group, (B) endometritis group. Beta-actin was used as an internal reference to normalize the quantitative data. The figure is a representative result, which was tested in each animal for one times with the similar result in four samples.

Fig. 6

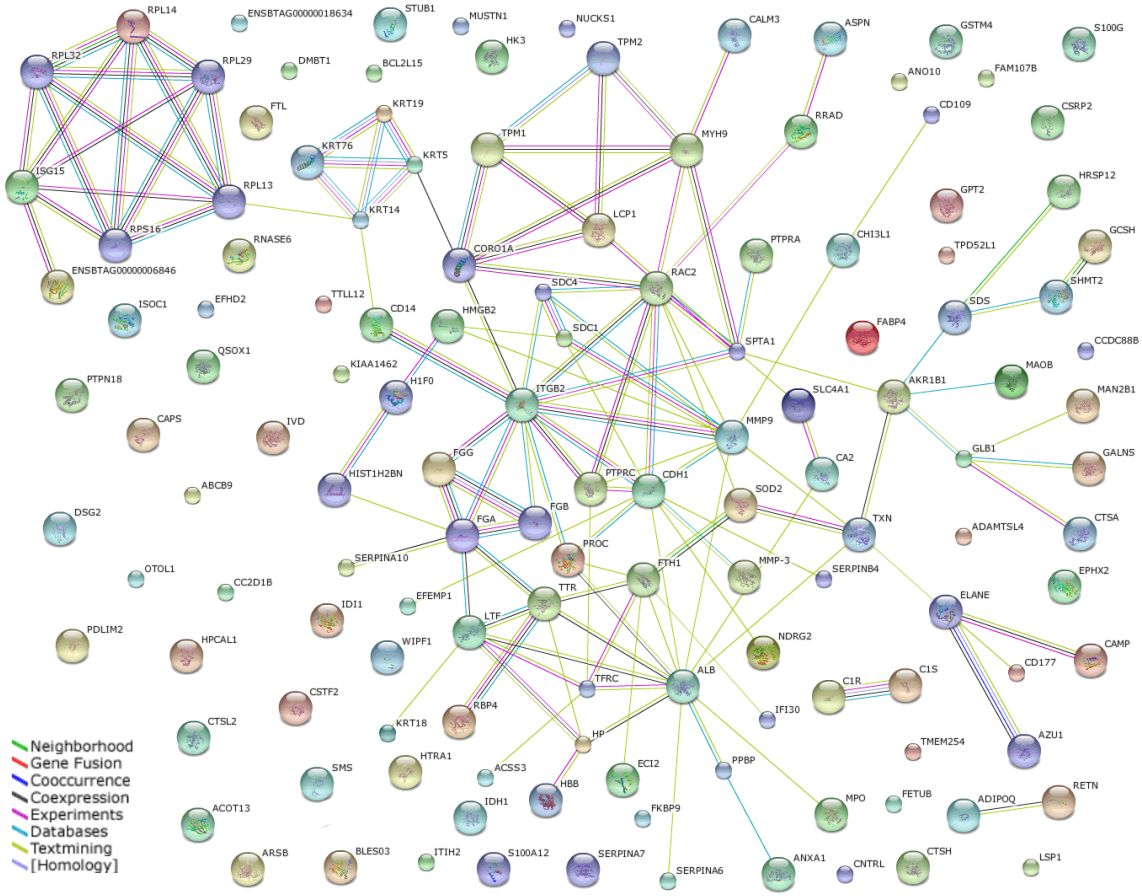

Figure 6. The protein-protein interaction network of DEPs from endometrial tissue following analysis by STRING software. An edge was drawn with 8 colorful lines as shown (see legend) which represent the existence of the eight types of evidence used in predicting the associations.