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Proteomic Analysis of Differentially Expressed Proteins in Bovine Endometrium with Endometritis

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Endometritis is one of the primary reasons for reproductive failure. In order to investigate endometritis-associated marker proteins, proteomic analysis was performed on bovine endometrium with endometritis. In bovine endometritis, desmin, α-actin-2, heat-shock protein (HSP) 27, peroxiredoxin-6, luteinizing hormone receptor isoform 1, collectin-43 precursor, deoxyribonuclease-I (DNase-I), and MHC class I heavy chain (MHC-Ih) were up-regulated. In contrast, transferrin, interleukin-2 precursor, hemoglobin β subunit, and potassium channel tetramerisation domain-containing 11 (KCTD11) were down-regulated in comparison to normal endometrium. The proteomic results were validated by semiquantitative-PCR and immunoblot analysis. The mRNA levels of desmin, transferrin, α-actin-2, HSP27, KCTD11, and MHC-Ih were up-regulated by over 1.5-fold, and showed a pattern similar to their proteomic profiles. Desmin and α-actin-2 protein showed positive correlations between proteomic analysis and immunoblot analysis. These results suggest that desmin and α-actin-2 may play important roles in endometritis-related function, and could be useful markers for the diagnosis of bovine endometritis.

Key Words: Actin, Desmin, Endometritis, Proteomics

INTRODUCTION

Endometritis is a major cause of reproductive failure in mammalian species. Endometritis is usually caused by infections of the inside wall of the uterus (endometrium). Secondary inflammation without systemic signs hinders the movement of sperm into the uterus and the implantation of the fertilized egg; thus, endometritis can cause uterine involution, subfertility, and infertility [1,2]. Normally, endometritis is diagnosed based on clinical examination. Several methods for diagnosing endometritis have been established using a variety of techniques, such as vasinoscopy combined with manual palpation of the uterus and cervix per rectum, cytology, ultrasonography, and metricheck [2-4]; however, subclinical endometritis remains undetected because it does not show any signs or symptoms. There is a consensus that subclinical endometritis has a significant negative impact on reproductive performance.

The endometrium shows dramatic morphological and secretory changes throughout the estrous cycle and during pregnancy. Such physiological changes reflect the extremely complex interactions between RNA and protein. Proteomic analysis and microarray are powerful tools for the identification of proteins and genes differentially expressed in many kinds of tissues and cells. Although many researchers have accumulated large amounts of data from various functional genomics and proteomics studies, little is known the proteome and genes that are affected in bovine endometritis. In this study, we performed proteomic analysis on samples of bovine endometrium with endometritis to find marker proteins that could indicate endometritis.

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METHODS

Samples

Korean cattle was maintained in free-stall facilities and Korean cattle (normal, n=3: endometritis, n=5; eight to ten years old) were used in this study. Endometritis is diagnosed based on clinical examination. Clear mucus with flakes of pus in the absence of an enlarged uterus can be regarded as signs for endometritis [5]. In this study, the uteri of Korean cattle were collected from a slaughterhouse and transported to the laboratory within 2 hrs on ice, in a RNA*later* solution (Qiagen, GmbH, Hilden, Germany), or in ice-cold phosphate-buffered saline. The endometrium

ABBREVIATIONS: DEPs, differentially expressed proteins; DNase-I, deoxyribonuclease-I; HSP, heat-shock protein; KCTD11, potassium channel tetramerisation domain-containing 11; MHC-Ih, MHC class I heavy chain.

was isolated from uteri with the same estrus cycle (ovary without corpus luteum). Endometria, with and without endometritis, were trimmed for the preparation of total RNA or protein. All experiments were performed with the approval of the Animal Ethics Committee of Gyeongsang National University.

Two-dimensional electrophoresis and image analysis

For two-dimensional electrophoresis (2-DE), proteins were isolated using the ReadyPrep Protein Extraction Kit (Soluble/Insoluble) according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Solubilized proteins (100 μg) isolated from bovine endometrium, with and without endometritis, were mixed with a rehydration solution, containing 8 M/l urea, 4% 3-[(3-cholamidopropyl)dimethylammio]-1-propane-sulfonate (CHAPS), 10 mM/l dithiothreitol (DTT), and 0.2% carrier ampholytes (desired pH values), and applied to immobilized pH gradient (IPG) strips (ReadyStrip, Bio-Rad) in a re-swelling tray (Bio-Rad). Several 7 cm- or 17 cm-long strips with pH gradients ranging from 3 to 10 and 17 cm-long strip gels with pH gradients ranging of 5 to 8 were used for the first screening and a sharper separation, respectively. The protein samples were rehydrated on dried IPG strips, and the proteins were separated by isoelectric focusing (IEF) using a Protein IEF Cell (Bio-Rad) at 250 V for 15 min, 10,000 V for 3 h, and then held at 10,000 V for a total of 90,000 Vh for the 17 cm-long strip. Following IEF, the strips were equilibrated with 0.395 M/l of Tris buffer (pH 8.8) containing 6 M/l urea, 2% SDS, 2% DTT, 20% glycerol, and 0.01% bromophenol blue for 20 min. The strips were equilibrated again with the same buffer containing 2.5% iodoacetamide instead of DTT. The proteins were separated in the second dimension by SDS-PAGE gel (12.5% polyacrylamide) electrophoresis. The resolved protein spots on the gels were visualized by silver staining. After staining, the 2-DE gels (n=4, each sample) were scanned in visible light at a resolution of 254 dots per inch using a Fluor-S MultiImager (Bio-Rad). The image data was analyzed using version 8.0 of the PDQuest 2-DE Gel Analysis Software. The background values were subtracted from the gel images. After automatic spot detection and matching, manual editing was performed on all spots to eliminate any artifact dots that had been matched incorrectly by the software. The normalized intensities of the individual protein spot were calculated as the intensities of a particular spot from the intensities of all the spots of the gel, and are expressed as PPM. The differentially expressed protein spots were compared to average normal quantities of gels from each sample.

Destaining and In-gel digestion of protein spots

The differentially expressed silver-stained spots in each sample were excised from the 2-DE gels and transferred into microcentrifuge tubes. The spots were destained with fresh chemical reducers in a 1 : 1 ratio of 30 mM/l potassium ferricyanide and 100 mM/l sodium thiosulfate until the brownish color of the spots disappeared. After destaining, the gel pieces were rinsed three times with distilled water to stop the destaining reaction, covered by ammonium bicarbonate (500 μl of 200 mM/l) for 30 min, dehydrated with 100 μl of acetonitrile (ACN), and then dried in a vacuum centrifuge. The gel pieces were rehydrated by covering them with a digestion buffer (50 mM/l NH_4HCO_3 and 5 mM/l $CaCl_2$) containing trypsin (12.5 ng/ml), and then incubated on ice for 45 min. The trysin-containing digestion buffer covering gels were replaced with 20 μl of a digestion buffer without trypsin. The gels with digestion buffer were incubated overnight at 37°C. The gel pieces were vigorously vortexed for 30 min after adding 20 μl ACN. The supernatants were transferred into clean microcentrifuge tubes and dried in a vacuum centrifuge. The resulting pellets were dissolved in 2 μl of 0.1% trifluoroacetic acid (TFA).

Matrix-assisted laser desorption ionization-time of flight- mass spectrometry (MALDI-TOF-MS)

For the matrix solution, α-cyano-4-hydroxycinnamic acid (40 mg/ml) was dissolved in 50% ACN and 0.1% TFA. The matrix and sample solutions (1 μl each) were mixed and loaded into the target wells, and then dried for 10 min at room temperature. After drying, the well was washed using de-ionized water and subjected to MALDI-TOF-MS analysis using a Voyager Biospectrometry Workstation (PE Biosystems, Foster City, CA, USA) with the following parameters: 20 kV accelerating voltage, 75% grid voltage, 0.02% guide wire voltage, 150 ns delay, and a mass gate from 800 to 3,500. The peptide mass fingerprints were analyzed using the program MS-Fit of ProteinProspect, developed by the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu). The NCBI and Swiss-Prot database of *Bos taurus* proteins was searched to identify the proteins, using monoisotopic peptide masses and allowing a molecular-mass range of 2-DE ±15%, a peptide mass accuracy of 50 p.p.m., and one partial cleavage.

Semi-quantitative RT-PCR

Total RNA from bovine endometrium was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Endometria, with and without endometritis, were homogenized in Trizol reagent using a homogenizer (FastPrep®-24, MP Biomedicals, OH, USA). The lysates were removed with chloroform and salted out with isopropanol and 70% ethanol. After centrifugation, the RNA pellet was dried, dissolved in diethylpyrocarbonate (DEPC) water, and quantified using a spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE, USA). First strand cDNA was synthesized from the total RNA (3 μg) isolated from the endometrium using oligo dT (SuperScript First-Strand Synthesis System, Invitrogen), and used as a template for PCR amplification with *Taq* polymerase (Takara Bio Inc, Otsu, Shiga, Japan). The cDNA was also quantified using NanoDrop. Specific primer sequences are listed in Table 1. PCR conditions included an initial denaturation at 94°C for 5 min, then 28 cycles at 94°C for 30 s, 55~60°C for 45 s, 72°C for 45 s, and a final extension step at 72°C for 10 min. RT-PCR products were separated by 1.2% agarose gel electrophoresis in a 0.5% tris-acetate (TAE) buffer and stained with ethidium bromide. The DNA fragments obtained by RT-PCR were directly sequenced with an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, CA, USA).

Western blot analysis

Bovine endometria were homogenized in extraction buffer (50 mM/l Tris-HCl, 0.1% SDS, 150 mM/l NaCl, 100μg/ml

Table 1. Primer sequences used for RT-PCR

Gene name	GenBank Acc. Nos.	Primer sequences (5'-3')	Expected size (bp)
Desmin	NM_001081575	Sense: GTGATGATCAAGACCATTGAG Antisense: GGTTCCTACATCCTACTCTGG	453
Transferrin	NM_177484	Sense: ATGGCTATACAGGGGCTTTCA Antisense: TTTGTGGAAAGTGCATGCTTC	488
α-actin	NM_001034502	Sense: CTGGACTTTGAGAATGAGATG Antisense: ATGATCTTGATCTTCATGGTG	329
IL-2	EF067918	Sense: CAAACGGTGCACCTACTTCAA Antisense: CAGCGTTTACTGTTGCATCATC	357
HSP27	NM_001025569	Sense: CTACATTTCCCGTTGCTTCAC Antisense: AAAAGAACATAGAGGTTTGG	300
Peroxi redoxin-6	BT020967	Sense: ACAATGGTGAAGAGCCCACA Antisense: GGGGTGTGTAGCGGAGGTAT	402
LH-receptor	AF491303	Sense: CGGGCTAGAGTCCATTCAGAC Antisense: GAGGACAGTCACATTTCCCGT	424
CL-43	BC116147	Sense: CCTTCAGGTGCCATGGGT Antisense: CTATTGTTGGGCTCCCCG	455
Hemoglobin β subunit	NM_173917	Sense: TTCCTTTAGTAATGGCATGAA Antisense: GCCCTTCATTTCTTTATGTCT	446
Potassium channel tetramerisation domain containing 11 (KCTD11)	BT021640	Sense: CGGACTTTTACCAGATCCGAC Antisense: CCCTAGTCTGCGGTACTCCAC	344
Deoxyribonuclease-1	NM_174534	Sense: TTACGACATCGTCCTCATCCA Antisense: TGCTGGACATCCAGGTAGACA	375
MHC class I heavy chain	XM_584616	Sense: CTGGAGAAGAGCAGAGATACA Antisense: GAAGTTTGTTCTGGAAGAGGT	428

phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% NP-40, and 0.5% sodium orthovanadate), incubated at 4°C for 30 min, and centrifuged at 13,000 rpm (16,609×g, Micro 17TR, Hanil, Korea) for 30 min (at 4°C). After centrifugation, the supernatants were collected and quantified by Bradford protein assay (Bio-Rad) as total proteins. The total proteins (30~100 μg/lane) were separated with a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (0.45 μm, Millipore, Bedford, MA, USA) in a buffer solution (TBS; 25 mM/l Tris-base, 190 mM/l glycine, and 20% methanol) using a semi-dry blotter (Bio-Rad). The transferred blot was stained with Ponceau S solution to check for effective homogeneous transfer. Destained blots were blocked with 5% fat-free milk and 0.05% Tween 20 in TBS for 1 h and immunoblotted with desmin monoclonal antibody (Abcam, Cambridge, CB4 0FW, UK), α-actin monoclonal antibody (Sigma-Aldrich, MO, USA), HSP27 monoclonal antibody (Stressgen, Michigan, USA), HSP70 monoclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA), and β-actin polyclonal antibody (Sigma). All primary antibodies were applied at 1 : 1,000 dilutions, at 4°C overnight. After binding of horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1 : 3,000; assay designs, Ann Arbor, MI, USA) at room temperature for 1 h, antigens were detected by enhanced chemiluminescence (ECL Plus kit; ELPIS, Taejeon, Korea) according to the manufacturer's protocol.

Data analysis for semi-quantitative PCR and western blot

LAS-4000 (Fujifilm corp, Tokyo, Japan), a luminescent image analyzer, captured images of the agarose gels and Western blots. The bands obtained from the RT-PCR and Western blot tests were quantified by Sigma Gel image analysis software (version 1.0, Jandel Scientific, CA, USA) or Quantity One software (version 4.6.3) attached to a GS-800 calibrated densitometer (Bio-Rad). Relative mRNA and protein levels were calculated by referring them to the amount of GAPDH and β-actin, respectively.

Statistics

Student's t-test was used with $p < 0.05$ as the criterion for significance. Data are represented as mean±SD.

RESULTS

Proteomic analysis of the bovine endometrium with endometritis

The soluble proteins isolated from bovine endometrium were proteomically analyzed to investigate differentially expressed proteins (DEPs) between normal samples and those with endometritis. The proteins were separated on pI 3~10 strips and SDS-PAGE, and the proteomic profiles of bovine endometrium with endometritis were compared to normal endometrium (eight samples were obtained from each different uterus). Representative 2-DE profiles of normal and endometritis samples are shown in Fig. 1A. The most abundant protein spots were enriched in the pI 4~8 regions. The proteins were further separated on pI 5~8 gel and stained with silver nitrate (Fig. 1B). The protein profile was similar between pI 3~10 and pI 5~8. Approximately 135 and 70 protein spots per silver-stained gel were observed in pI 3~10 and pI 5~8, respectively, by the naked eye, and 43 spots were recorded by the PDQuest program. The

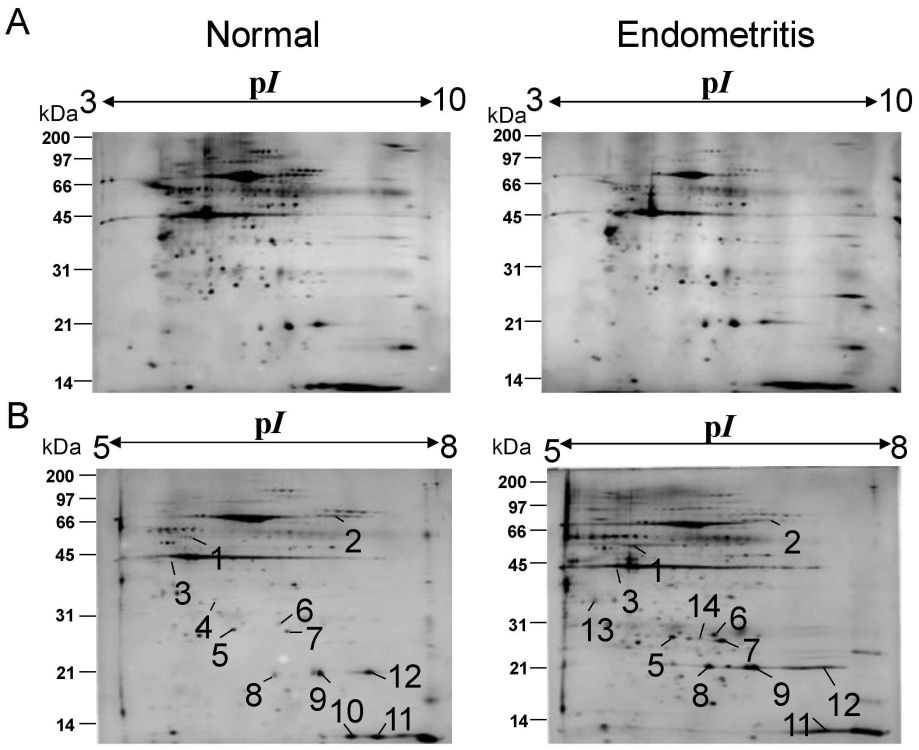

Fig. 1. Two-dimensional gel electrophoresis of bovine endometrium (A, B) Representative two-dimensional gel electrophoresis profiles of endometrium obtained from Korean cattle with and without endometritis. The soluble protein (100 μg) of each isolate was loaded on the 2-DE gel. The 2-DE gels were focused on 17-cm IPG strips with immobilized pH ranging 3 to 10 (A) or 5 to 8 (B). Proteins were then separated on 12.5% polyacrylamide gel. Molecular size markers are shown on the left-hand side of the gels. The proteins were visualized by silver staining. Number (1 to 14) represents DEP identified by PDQuest.

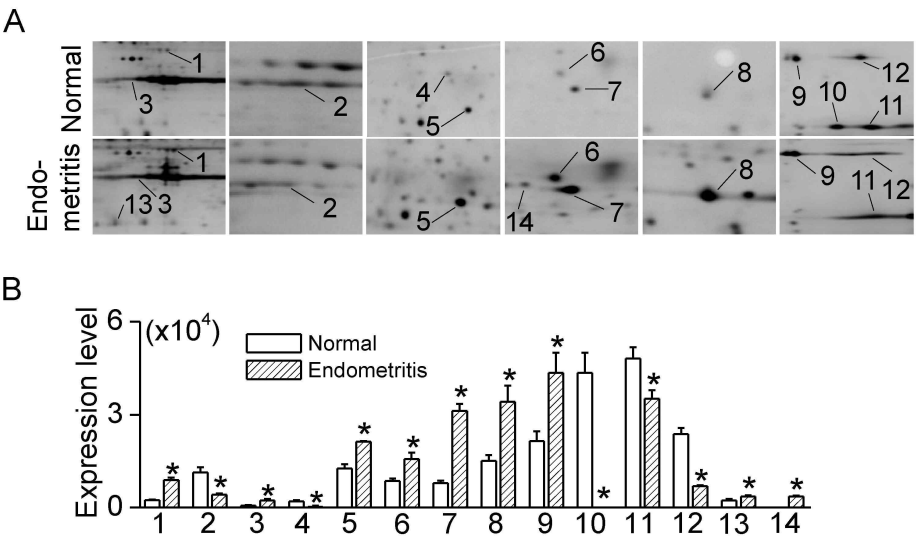

Fig. 2. Differentially expressed proteins in bovine endometrium with endometritis (A) Details of 2-DE gel images showing selected DEPs. The enlarged images of DEPs contain some additional protein spots. (B) Summary of intensities of DEP spots. Each bar represents mean±SD of four repeated experiments. The asterisks indicate the significant difference from the corresponding control value obtained for normal endometrium ($p < 0.05$).

numbers of protein spots observed by the naked eye were represented by counting strong spots. Fig. 2A shows detailed images of DEPs as enlarged images of Fig. 1B. Their protein intensities were summarized in Fig. 2B. As shown in Fig. 2A, of 43 spots recorded by the PDQuest, 28 spots (including those that were overlapping or low-intensity) were selected and identified by peptide mass fingerprinting using MALDI-TOF-MS. The intensities of these 28 spots were changed in endometrium with endometritis. The 12 observed proteins (No. 1 to 14) identified by MALDI-TOF-MS and database-searching were desmin, serotransferrin precursor (transferrin), aortic smooth muscle actin (α-actin-2), interleukin-2 precursor (IL-2), heat-shock protein β-1/heat shock 27 kDa protein (HspB1/HSP27, referred to as HSP27 from here on), peroxiredoxin-6 (PRSIN-6), luteinizing hormone receptor isoform 1 fragment (LH1F), collectin-43 precursor (CL-43), hemoglobin β subunit (Hg_3), potassium channel tetramerisation domain-containing 11 (KCTD11), deoxyribonuclease-I (DNase-I), and major histocompatibility complex (MHC) class I heavy chain (MHC-Ih) fragment. Of these, eight proteins (desmin, α-actin-2, HSP-27, PRSIN-6, LH1F, CL-43, DNase-I, and MHC-Ih) were up-regulated,

whereas four proteins (transferrin, IL-2, Hgβ, and KCTD11) were down-regulated in the bovine endometrium with endometritis. Table 2 summarizes the up- and down-regulated proteins. The rest of the identified proteins (14 of 28 spots) were hypothetical proteins (Table 3). Ubiquitination-related proteins were detected in endometrium. The MHC-Ih (spot No. 14) showed a 27 kDa size fragment. The difference in isoelectric points (p*I*) of DEPs identified in this study, especially HSP27 and Hgβ detected in two spots with different p*I*, from p*I* calculated from the reported primary structure may result from occurrence of post-transcription, translation modifications, or alternative splicing of transcripts (Fig. 1B and Table 2).

Confirmation by semi-quantitative PCR and Western blot analysis

In the 2-DE analysis, many proteins were up- or down-regulated. Notably, physiological changes are a reflection of extremely complex interactions between gene and protein. In order to validate 2-DE data through an additional examination of gene levels, semi-quantitative RT-PCR was performed using first-strand cDNA prepared from bovine endometrium. Specific primers were designed to am-

Table 2. List of differentially expressed proteins identified by MALDI-TOF-MS in the endometrium with endometritis

Spot No.	Annotation	MW /pI	Swiss-Prot. Acc. Nos.	CV (%)	Fold change	MOWSE score	% Cov
1	Desmin	53.4/5.2	O62654	24.8/33.9	3.7 (△)	558,976	43.7
2	Serotransferrin precursor (Transferrin)	77.8/6.8	Q29443	58.9/37.9	0.37 (▽)	387	21.7
3	Actin, aortic smooth muscle (α-actin-2)	42.0/5.2	P62739	0/47.9	1.53 (△)	10,758	42.2
4	Interleukin-2 precursor (IL-2)	17.6/6.1	P05016		- (▽)*	228	18.1
5	Heat-shock protein β-1 (HSP27)	22.4/6.0	Q3T149	26.7/31.4	1.68 (△)	123,678	45.9
6	Peroxiredoxin-6	24.9/6.0	O77834	43.4/23.9	1.82 (△)	269	26.9
7	Heat-shock protein beta-1 (HSP27)	22.4/6.0	Q3T149	10.8/8.2	3.93 (△)	35,712	44.8
8	Luteinizing hormone receptor isoform 1 (Fragment)	24.7/5.2	Q28026	43.2/22.6	2.27 (△)	931	36.8
9	Collectin-43 precursor (CL-43)	33.6/5.0	P42916	28.9/12.2	2.3 (△)	194	28.4
10	Hemoglobin β subunit	16.0/7.0	P02070	10.4/0	- (▽)*	226	51.7
11	Hemoglobin β subunit	16.0/7.0	P02070		0.73 (▽)	159	44.1
12	Potassium channel tetramerisation domain containing 11 (KCTD11)	20.5/6.4	Q58DF7		0.71 (▽)	822	36.3
13	Deoxyribonuclease-1	31.3/5.3	P00639		+ (△)†	222	21.7
14	MHC class I heavy chain (Fragment)	40.4/5.4	O78186	0/56.4	+ (△)†	23,348	42.5

*Undetected in endometritis, †Undetected in normal endometrium.

Table 3. List of hypothetical proteins identified by MALDI-TOF-MS in the endometrium with endometritis

Hypothetical ID	Homological protein name	Function	Expression level
MGC3234	Hypothetical protein MGC3234	Secreted protein	△
MGC11335	Glucose-fructose oxidoreductase domain containing 2 (GFOD2)	Promotes matrix assembly	△
MGC126924	Dipeptidase 1 precursor (renal)	Hydrolyzation of dipeptides/Renal metabolism of glutathione/ Regulation of leukotriene activity	△
MGC127104	Vacuolar protein sorting-associated protein 28 homolog	Component of the ESCRT-I complex, a regulator of vesicular trafficking process	△
MGC127130	RING finger protein 186	Ubiquitin protein	△
MGC127165	GTP-binding protein SAR1b	Transport from the endoplasmic reticulum to the Golgi apparatus/ Activated by the guanine nucleotide exchange factor PREB/ Selection of the protein cargo and the assembly of the COPII coat complex	▽
MGC128032	Proteasome subunit beta type-3	A multicatalytic proteinase complex/An ATP-dependent proteolytic activity	▽
MGC128156	Beta-enolase	A function in striated muscle development and regeneration	△
MGC128193	MIF4G domain-containing protein	Functions in replication-dependent translation of histone mRNAs	▽
MGC128394	Hydroxyacid oxidase 2	2-hydroxyacid oxidase activity	△
MGC128422	Sphingolipid delta(4)-desaturase DES1	-	▽
MGC128631	Stromal cell-derived factor 2 precursor	Ubiquitously expressed in various cancer cell lines	△
MGC128818	F-box only protein 6	One of the four subunits of the ubiquitin protein ligase complex	△
MGC129042	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	Regulation of cell division, glycogen metabolism, muscle contractility, and protein synthesis	△

plify short DNA fragments (Table 1). Of the 12 proteins identified in 2-DE analysis, PCR products for desmin, transferrin, α-actin-2, HSP27, PRSIN-6, KCTD11, and MHC-Ih were obtained and confirmed by sequencing in both normal and endometritis samples (Fig. 3A). PCR products of IL-2, LH1F, CL-43, Hg3, and DNase-I were not detected using cDNAs prepared from endometria, with or without endometritis, despite multiple attempts (n=10). Consistent with 2-DE data, semi-quantitative PCR analysis showed an up-regulation of desmin, α-actin-2, HSP27, and MHC-Ih mRNA expression; this PCR analysis showed a down-regulation of transferrin and KCTD11 in the endometrium with endometritis (Fig. 3A); however, PRSIN-6 did not show a positive correlation between protein expression and mRNA expression (Fig. 2 and Fig. 3A).

In order to verify the 2-DE data, we also performed western blot analysis with the antibodies against desmin, HSP27, and α-actin-2, demonstrating a high MOWSE score. MHC-Ih was ruled out for detection because it is highly-regulated during inflammation. In agreement with 2-DE data, desmin and α-actin-2 were significantly increased in the endometrium with endometritis, compared to normal endometrium (Fig. 3B). As shown in Fig. 3C, HSP27 showed a trend contrasting with that of the 2-DE data. In the samples with endometritis, HSP27 was down-regulated; however, two bands demonstrated up-regulation in the endometritis samples, at 50~60 kDa and 70 kDa, using antibodies for anti-HSP27 purchased from two different companies (Stressgen and Santa Cruz). This result suggests that other HSPs might be present in the bovine endometrium; however, we were not able to read the sequence of this 50~60 kDa protein due to impurity. A monoclonal antibody against HSP70 was used to detect a 70 kDa band. Western blot analysis showed that HSP70 was up-regulated in the endometritis samples (Fig. 3C). However, there was no significant difference in HSP expression levels between normal and endo-

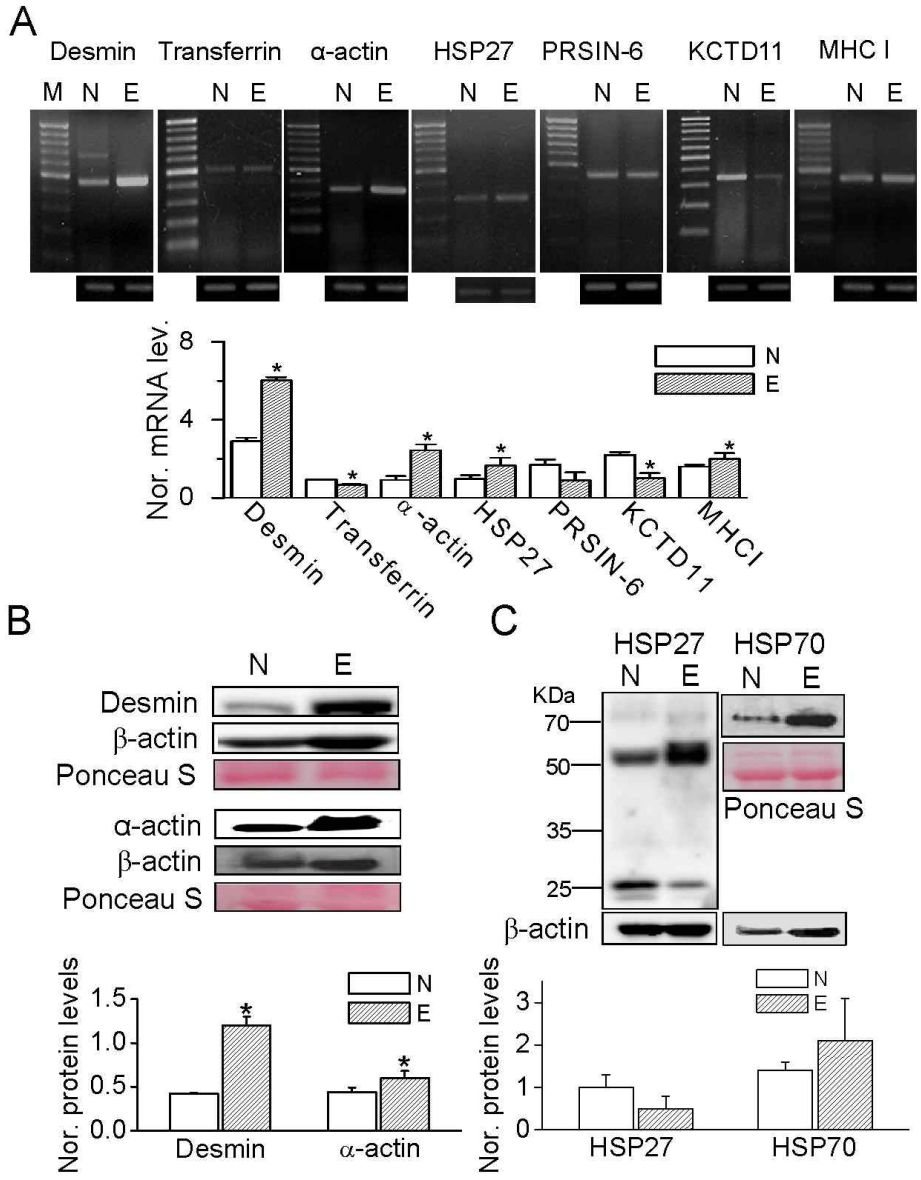

Fig. 3. Validation of 2-DE analysis by semi-quantitative PCR and western blot analyses in bovine endometrium, with and without endometritis. (A) RT-PCR products for desmin (453-bp), transferring (488-bp), α-actin (329-bp), HSP27 (300-bp), PRSIN-6 (402-bp), KCTD11 (344-bp), and MHC-I (428-bp) derived from bovine endometrium. The first lanes show the 1-kb DNA ladder. Bar graph shows normalized mRNA levels of desmin, transferrin, α-actin, HSP27, PRSIN-6, KCTD11, and MHC-I in the endometria with and without endometritis. The expression levels were normalized to GAPDH. Each bar represents mean±SD of five repeated experiments. The asterisks indicate the significant difference from the corresponding control value obtained for normal endometrium ($p<0.05$). N and E represent normal endometrium and endometrium with endometritis, respectively. (B, C) Western blot analyses of desmin, α-actin, HSP27 and HSP70 proteins. Ponceau S staining of total proteins served as a loading control. The bar graph shows the up-regulation of desmin and α-actin in endometrium with endometritis. Each bar represents the mean±SD of three repeated experiments. Asterisks indicate the significant difference from the corresponding control value (normal), $p<0.05$.

metritis. As shown in Fig. 3B and 3C, the protein levels of β-actin as well as α-actin were also up-regulated. However, the alteration in β-actin expression level was lower than that in α-actin expression. Unlike actin, α-tubulin expression levels were similar between normal endometrium and endometrium with endometritis (data not shown). The β-actin was used as the internal reference protein. Although β-actin expression level is changed in endometrium with endometritis. the rate of change is smaller than that of α-actin. Ponceau S red staining of the blotted membrane showed that almost equal amounts of proteins between normal and endometritis were applied for Western blot analysis. These results indicate that endometritis might modulate actin cytoskeletal protein expression.

DISCUSSION

This study demonstrates for the first time that desmin, α-actin-2, HSP27 (HSP70), and MHC-Ih proteins are substantially up-regulated in endometria with endometritis. In particular, this study gives meaning to identification of proteins with similar profile to mRNA expression level despite we detected fewer protein spots than those of other studies. We counted and analyzed protein spots expressed in gels strongly. Berendt et al. [6] detected approximately 1,000 spots in bovine endometrium. Methods of protein extraction and detection, sample condition, and endometrial cycle are likely to result in difference in the number of protein spots. Of the 12 proteins identified in this study, desmin, transferrin, α-actin-2, HSP27, KCTD11, and MHC-Ih showed consistency between protein and mRNA expression. In particular, desmin, α-actin-2, HSP27, and MHC-Ih showed high MOWSE scores. These proteins (except MHC-Ih) were validated by immunoblot analysis. Desmin and α-actin-2 proteins, demonstrating up-regulation in endometritis through 2-DE results, showed an increase in immunoblot analysis; HSP27 showed down-regulation in endometritis, contradicting the 2-DE results; however, we observed an up-regulation of HSP70 in endometritis by immunoblot analysis, which was not detected in the 2-DE analysis. In the HSP27 and PRSIN-6, negative correlations from 2-DE, RT-PCR, or immunoblot analysis may result from difference in the rates of transcription, translation, protein turnover or immune reaction. We focused on describing the roles of desmin, α-actin, HSPs, and MHC-I in the apoptosis and immune response, which could be elicited by endometritis.

Differentially expressed proteins in endometritis are related to apoptosis and immune response

The regulation of inflammatory and immune response to endometritis may assist treatment of endometritis. In the endometritis, up-regulated peripheral blood neutrophil produces reactive oxygen species (ROS), and the ROS and cytokines could induce or suppress apoptosis of endometrial cells.

Many cytoskeletal proteins, including desmin and actin, could act as substrates in cells undergoing apoptosis. Desmin is a major target of oxidation and nitration, which have toxic effect [7]. The α-actin-2 was found in myoepithelial cells and myofiboblasts of pathologic tissues [8]. Enriched HSPs expression has been shown to play an important role in the protection against apoptosis by suppressing ROS generation, mediating the MAP kinase pathway, and inhibiting cytochrome c release, caspase activation, and Fas-induced apoptosis [9,10]. In the human polycystic ovarian syndrome ovaries (POSO), HSP27 expression was decreased, indicating that POSO may suffer cell apoptosis because of down-regulation of HSP27 [10]. In addition to HSPs, other apoptosis-related proteins, such as transferrin, PRSIN-6, Hg_3, KTDC11, and DNase-I were regulated in bovine endometritis (Fig. 2 and Table 2). Endometritis-induced apoptosis may be prevented by decreasing pro-apoptotic and increasing anti-apoptotic signals. Severe apoptosis was not detected in endometritic samples by TUNEL staining, suggesting that the DEPs might exhibit strong protective effects against apoptosis (data not shown).

The MHC-I molecules function in immune responses, and are primarily involved in the discrimination of self from non-self. The immune response to the implanting embryos plays important roles in pregnancy success. It is generally accepted that modification or down-regulation of MHC-I molecules in placental tissues is beneficial to pregnancy in mammalian species [11,12]. Up-regulation of MHC-I in endometritis may result in low fertility and pregnancy rates; however, the expression of MHC-I could depend on steroid hormone levels. Low fertility and pregnancy rates by endometritis may be due to dysfunction of steroid hormone and modulation of proteins regulated by sex steroid hormone. In addition to MHC-Ih, immune-related proteins, such as IL-2 and CL-43 were regulated in bovine endometritis.

Desmin, α-actin-2, HSPs, and MHC-I may regulate apoptosis and immune response in bovine endometritis in interaction with one another or other proteins

Desmin is a major intermediate filament (IF) that is a part of the cytoskeleton expressed in skeletal, smooth, and cardiac muscle tissues, and is regarded as a muscular marker [13,14]. Actin is a ubiquitous cytoskeletal protein, which is essential for multiple cellular functions, and is expressed in all eukaryotic cells [15]. Levels of these cytoskeletal proteins are modified in the processes of inflammation and apoptosis [16,17]. When cells are exposed to stress, such as inflammation and apoptosis, the cells show modifications of the different cytoskeletal networks and increase HSP synthesis [15]. The expression of HSPs, which are well-known stress response proteins, is substantial during pathophysiological stress conditions. Furthermore, HSPs increase cell survival by protecting and disaggregating stress-labile proteins [18]. Several HSPs have been reported to interact with the different cytoskeletal components. Primarily, large HSPs (such as HSP90 and HSP70) bind to the microtubule network and centrosome, whereas small HSPs (such as HSP27) seem to be involved in maintaining the integrity of actin and the IF [15,19]. The actin cytoskeleton is protected by small HSPs against disruptions induced by various stressful conditions [15]. Desmin, α-actin-2, HSP27, and HSP70 up-regulated in endometritis are involved in the regulation of a pathological condition induced by inflammation (Fig. 2 and 3). MHC, which is a group of molecules known as the immunoglobulin supergene family, also interact with cytoskeletal actin [20]. Their up-regulation probably plays a more important role in the processes of endometritis than under normal conditions, since endometritis induces a complex array of in vivo changes. Interestingly, we found that both α-actin and β-actin were up-regulated in endometritis (Fig. 3B and C). These actins could be regulated by the endocrine environment; they could respond to estrogen and progesterone, which modu-

late the uterine gene expression responsible for uterine remodeling prior to implantation and placentation [21,22]. HSP27 and HSP70 highly expressed in organs of the human female reproductive tract are also regulated by estrogen and progesterone secreted in the human and murine endometrium during the different phases of the menstrual cycle [23,24]. The sex steroid hormones also regulate both systemic and local immune responses. Serum estrogen and progesterone levels were changed with the severity of endometrial inflammation [25]. Actin, HSP, and MHC-I proteins regulated by sex steroid hormone play important roles in the processes of endometritis. Further studies will be needed to identify their interactions with other proteins, as well as the specific up-regulation mechanism elicited by stressful conditions and to confirm the relationship between steroid hormone levels and these proteins. In addition to being well-known as markers for decidual cells, muscle tissues, and inflammation, desmin, actin, HSP, and MHC-I were expressed in the bovine endometrium and were up-regulated in endometritis. Hypothetical proteins differentially expressed in the endometrium with endometritis could also be considered as a diagnostic marker for endometritis. Ubiquitin proteins are likely to be involved in a variety of disease processes.

In conclusion, these results suggest that desmin, α-actin, HSP27, HSP70, and MHC-I may play important roles in preparing the endometrium for implantation. This study provides developing diagnostic markers, which are useful for comparisons of early and later terms of endometritis, and helps to establish a bovine endometritis proteome database that will broaden our understanding of the pathogenesis of endometritis.

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