Quantitative proteomic analysis shows involvement of the p38 MAPK pathway in bovine parainfluenza virus type 3 replication

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Abstract
Background: Bovine parainfluenza virus type 3 (BPIV3) infection often causes respiratory tissue damage and immunosuppression and further results in bovine respiratory disease complex (BRDC), one of the major diseases in dairy cattle, caused huge economical losses every year. However, the pathogenetic and immunoregulatory mechanisms involved in the process of BPIV3 infection remain unknown. However, the pathogenetic and immunoregulatory mechanisms involved in the process of BPIV3 infection remain unknown. Proteomics is a powerful tool for high-throughput identification of proteins, which has been widely used to understand how viruses interact with host cells.

Methods: In the present study, we report a proteomic analysis to investigate the whole cellular protein alterations of MDBK cells infected with BPIV3. To investigate the infection process of BPIV3 and the immune response mechanism of MDBK cells, isobaric tags for relative and absolute quantitation analysis (iTRAQ) and Q-Exactive mass spectrometry-based proteomics were performed. The differentially expressed proteins (DEPs) involved in the BPIV3 invasion process in MDBK cells were identified, annotated, and quantitated.

Results: A total of 116 proteins, which included 74 upregulated proteins and 42 downregulated proteins, were identified as DEPs between the BPIV3-infected and the mock-infected groups. These DEPs included corresponding proteins related to inflammatory response, immune response, and lipid metabolism. These results might provide some insights for understanding the pathogenesis of BPIV3. Fluorescent quantitative PCR and western blotting analysis showed results consistent with those of iTRAQ identification. Interestingly, the upregulated protein MKK3 was associated with the p38 MAPK signaling pathway.

Conclusions: The results of proteomics analysis indicated BPIV3 infection could activate the p38 MAPK pathway to promote virus replication.

Keywords: Bovine parainfluenza virus type 3 (BPIV3), Differentially expressed proteins, p38 MAPK signaling pathway, Quantitative proteomics

Introduction
Bovine parainfluenza virus type 3 (BPIV3) is an enveloped, single-stranded negative-sense RNA virus that belongs to the family Paramyxoviridae, genus Respirovirus [1]. BPIV3 infection results in pneumonia and atypical interstitial pneumonia in cattle and leads to severe secondary bacterial infection and other related clinical symptoms. BPIV3 infection and other viral or bacterial infections often cause bovine respiratory disease complex...
BPIV3 infection remains largely unclear. To investigate the changes in the phospholipid system during the process of viral infection, iTRAQ mass spectrometry (MS) was performed.

The iTRAQ quantitative proteomics technique has been widely used to study interaction between virus and host based on high sensitivity and quantitation accuracy [8]. An et al. used iTRAQ to determine the differentially expressed proteins (DEPs) of transmissible gastroenteritis virus (TGEV)-infected PK-15 cells, which identified 60 upregulated and 102 downregulated proteins in the TGEV infection process. Their analysis revealed that many upregulated proteins were associated with interferon signaling and that TGEV infection could activate the JAK-STAT1 signaling pathway [9]. In order to provide a scientific basis for the PEDV pathogenesis, the iTRAQ quantitative proteomics technique identified the proteins associated with porcine epidemic diarrhea virus (PEDV) infection [10]. Isobaric tags for relative and absolute quantification (iTRAQ) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) approaches have been used to provide the proteomic expression profiles of host cells in response to infections by various viruses, including classical swine fever virus [11], porcine deltacoronavirus [12], influenza A (H1N1) virus [13], and porcine rotavirus [14]. iTRAQ coupled with LC-MS/MS analysis is a robust quantitative proteomics technique for the comprehensive analysis of differentially expressed proteins (DEPs). In the present study, the DEPs in BPIV3-infected MDBK cells were identified and quantitatively analyzed for the first time by the iTRAQ-based proteomics approach. MDBK cells have been selected for use in many studies [15, 16]. Usually MDBK cells are not only used for the isolation, propagation, and basic studies of BPIV3, but also as host for many other bovine pathogens, such as bovine respiratory syncytial virus (BRSV) and bovine herpesvirus type 1 [17, 18].

The expression levels of 116 proteins were found to be significantly altered after 24 h of BPIV3 infection. These cellular DEPs were assigned to several biological processes according to bioinformatics analysis. These changes activated the p38 MAPK pathway promoted the BPIV3 replication, providing a global understanding of the host action with BPIV3 infection.

Materials and methods

Virus infection of MDBK cells

MDBK cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) medium containing 10% fetal bovine serum (FBS) and 100 g /ml penicillin and 100 g /ml streptomycin. Cell culture conditions at 37 °C with 5% CO₂ in 24 h. The BPIV3 DQ strain (GenBank accession no. HQ462571) was isolated and identified in the preventive veterinary laboratory of Heilongjiang Bayi Agricultural University. MDBK cells were infected with BPIV3 at multiplicity of infection (MOI = 1). Uninfected cells were used as mock-infected groups. Each experiment was carried out with three replicates. The cytopathic effect (CPE) was observed and the growth curve of BPIV3 was measured. TCID₅₀ were measured by the Reed-Muench method.

Protein isolation, digestion, and labeling with iTRAQ reagents

All the cell samples, including BPIV3-infected group and control group, were cleaned with cold PBS twice and centrifuged at 1000 g at 4 °C for 10 min to harvest cells. Then, the collected cells were lysed to extract proteins in the 300 μL SDT (1 mM PMSF, 2 mM EDTA and 10 mM DTT). The dissolved protein samples were harvested with centrifugation at 1 4000 g for 40 min at 4 °C. The concentration of the protein supernatant was determined using BCA protein assay. The protein 100 μg was digested for 8 h at 37 °C by the sequencing-grade modified trypsin. The protein samples were labeled by different iTRAQ tags on the basis of iTRAQ Reagent-8plex Multiplex Kit instruction (AB SCIEX). Three mock-infected samples were labeled by iTRAQ 113, iTRAQ 114 and iTRAQ 115, respectively; three BPIV3-infected samples were labeled by iTRAQ 116, iTRAQ 117 and iTRAQ 118, respectively. Then the labeled samples were mixed and dried by using vacuum concentrator.
LC–MS/MS analysis
The labeled peptide samples were purified and separated by AKTA purification system. The operation methods and solution preparation were performed essentially as described previously [19]. The whole elution process was monitored at 214 nm and collected every minute. Thirty distillates were collected and neutralized in 10 pools and desalinated in a C18 cartridge. After each fraction was vacuum centrifuged, the sample was dissolved in 40 μL 0.1% trifluoroacetic acid and kept frozen at −80 °C for mass spectrometry analysis. Each sample was separated by capillary high-performance liquid chromatography (Thermo scientific EASY column (2 cm, 100 μm 5 μm, C18)). The chromatography conditions were as follow: Water with 0.1% formic acid (A) and Acetonitrile with 0.1% formic acid (B) as mobile phase. The flow rate was 300 nL per minute and the mobile phase gradient program was used: 0–33 min, from 0 to 40%(B); 33–34 min, from 40 to 100%(B); 34–35 min maintained 100% and then back to 40%. Then, proteins were analyzed by using a Q-Exactive mass spectrometry (Thermo Finnigan) at positive ion mode (parameters: mass range: 300–1800 m/z; Dynamic exclusion: 40.0 s, MS2 Activation Type: HCD, Normalized collision energy: 30 eV).

Database search and bioinformatic analysis
MS/MS data were searched in the bovine subset database from the UniProt database (release March 22, 2016, containing 32 015 sequences) and proteins were identified by Mascot 2.3.02. The peptide for quantification was automatically selected by Paragon™ algorithm to calculate the reporter peak area, error factor (EF) and p-value. The proteins expression levels in BPIV3-infected cells were calculated to compare with those of mock-infected cells. Proteins with fold changes > 1.5 and p-values < 0.05 were considered as significantly different expressions. Auto bias-corrected were executed to decrease artificial error. These proteins were further classified by Gene Ontology (GO) and pathway enrichment analysis (http://www.geneontology.org).

RNA extraction and real-time PCR analysis
The mRNA levels of differentially expressed proteins were analyzed by real-time PCR. Total RNA of the MDBK cells in the BPIV3 infected group and the control group was extracted by TRIZol reagent (Takara) according to the manufacturer’s protocol. The RNA concentration was measured using NanoDropnd-1000. Agarose gel electrophoresis detected the total RNA 1 μL. The cDNAs of these samples were obtained by reverse transcription. Relative quantitative real-time PCR was performed in a 25 μL system that containing 12.5 μL SYBR Premix Ex TaqTM II, 2 μL primers, 2 μL cDNA samples and 8.5 μL water. The reaction condition was 95 °C for 10 min, then 40 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. The melting curves were obtained. The gene of GADPH was used as the internal reference gene. All of the primers were used in the PCR tests shown in Table 1. The data statistic was based on three independent experiments.

Western-blot
The infected MDBK cells were washed two times with PBS and disrupted with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1% Triton X-100, supplemented with 1 tablet of Complete-Mini Protease Inhibitor Cocktail per 50 ml buffer). The cell lysates were centrifugated at 12,000 × g for 10 min to harvest supernatants. Protein assays were performed on all supernatants using the Bradford method. For Western blot analysis of the whole-cell lysates, samples, each containing 25–30 μg of protein equivalent, were dissociated in SDS-PAGE loading buffer and separated by 12% gradient SDS-PAGE. Proteins were then transferred to an Immobilon-FL membrane (Millipore). The primary antibodies, including MKK3 (rabbit, Cell Signal Technology5674, Danvers, MA), p38 phosphorylation (p-38) 1:1000 (mouse, Cell Signal Technology9216, Danvers, MA), β-actin 1:10,000 (mouse, Sigma), were incubated on the membrane at 4 °C over-night. As a secondary antibody, goat anti-rabbit and goat anti-mouse immunoglobulin G (1:1000, Santa Cruz Biotechnology Inc.) was applied to the membrane at 4 °C for 1 h. After further washes, the immune complexes were revealed by enhanced chemiluminescence by the ECL detection kit (Beijing Biosea Biotechnology Co., Ltd.).

Statistical analysis
Statistical analysis was performed in Microsoft Excel for two-tailed Student's t test or one-way analysis of variance (ANOVA). The p-values < 0.05 were considered statistically significant.

Results
Detection of the BPIV3 activity in MDBK cells
To determine the optimal sampling time point for proteomics analysis after BPIV3 infection, MDBK cells were cultured in a monolayer and inoculated with BPIV3. At different time points 0, 6, 12, 18, 24, 36, and 48 h post inoculation, the cell-virus suspension was harvested and the CPE was observed (Fig. 1A). The TCID₅₀ was measured. The growth curve of BPIV3 was plotted according to the results of TCID₅₀ which showed that BPIV3...
proliferated rapidly from 24 to 36 h after infection, indicating active intracellular replication of the virus (Fig. 1B).

The MDBK cells were inoculated with BPIV3 at the dose of 1 multiplicity of infection (MOI = 1), and CPE was observed at different time points after infection.
The results showed that lesions began apparently at 12 h after BPIV3 infected the cells, and then, became more worse with time (Fig. 1A). The viral titer reached a peak of approximately 5.7 at 36 h and then gradually and continuously declined (Fig. 1B). Generally, the optimal time for a proteomic analysis is when viral replication remains high but no significant host cell cytoskeleton or membrane rearrangement is observed [20]. According to the post-infection cytopathic conditions combined with virus proliferation, cells infected at 24 h were used as the time point for proteomics analysis.

**Protein profiling and iTRAQ quantification**

The collected protein samples of BPIV3-infected and mock-infected MDBK cells were labeled with iTRAQ reagent in three biological replicates. The quantitative information of the two experimental group ratios (ratio [infection/control]) was obtained by integrating the peptide segment information of three biological duplicates in the mock-infected group (control) and the BPIV3-infected group (infection).

The changes in the protein expression level between the two groups were analyzed based on statistical significance. A total of 2804 proteins were detected and quantified by LC–MS/MS. 116 proteins significantly changed according to $P < 0.05$ (Fig. 2) and the proteins change ratio of $\geq 1.5$. Among these proteins, 74 proteins were significantly upregulated and 42 proteins were markedly downregulated (Table. 2). The most significantly upregulated protein was vesicle-related membrane protein, which is related to autophagy. The most significantly downregulated protein was the integrin complement protein, which is a receptor protein of viral infection (Table. 2).

**GO annotations of the DEPs**

GO annotations for DEPs. The proteins were annotated into three major categories: biological process (BP), cellular component (CC), and molecular function (MF) (Fig. 3). The GO enrichment analysis in the biological process showed that the DEPs were significantly enriched in five processes, including single organism process, response to stimulus, metabolic process, cell process, and biological regulation. The proteins involved in the biological regulation process were found most, followed by those involved in the stimulation response process. In this study, the proteins in the stimulation response process mainly included tyrosine phosphatase, signal transduction protein 1, Rab5 GDP/GTP conversion factor 1, interleukin-13 (IL-13), mitogen-activated protein kinase 7 (MAPK7), FOX transcription inhibitory factor 3 (Foxp3), calcium phosphate, protein tyrosine phosphatase protein receptor, MAP3K10, human telomerase reverse transcriptase, and SSNA1. IL-13 is the most important inflammatory factor that causes airway inflammation. It plays a key role in the occurrence of chronic airway inflammatory disease, which induces high secretion of mucus. Foxp3 is a member of the Fox transcription factor family that plays an important role in maintaining the immune function of the body [21]. The DEPs in BPIV3-infected MDBK cells may cause the initial cellular stress response. The precise role of these DEPs in the BPIV3 infection process need to be further investigated.

**Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis of the DEPs**

The KEGG pathway database is a collection map based on the molecular interaction pathways and cellular response networks. The DEPs were identified and mapped to six KEGG pathways, including metabolism, cellular processes, organismal systems, environmental information process, genetic information process, and disease pathways. The organismal systems and disease pathways were enrichment pathways, represented by 37 and 43 pathway groups, respectively.

In the metabolic pathways, the DEPs participated in 13 pathways related to the metabolism of glucose, lipid, amino acid, and nucleotides (Fig. 4A). These pathways affect the metabolism of three major nutrients in cells. The cellular processes involved 10 pathways (Fig. 4B), including the Focal adhesion pathway and the Phagosome pathway, both of which were involved in the viral infection process. The integrin protein was the key protein in these two pathways. The lysosome pathway, phagosome
### Table 2 The DEPs lists between BPIV3-infected group and mock group

| No. | Protein name                  | Uniprot Accession no | GO annotation | P value | V/C  |
|-----|-------------------------------|----------------------|---------------|---------|------|
| 1   | Integrin beta                 | Q6PT99               | Single organismal cell–cell, adhesion-respons to stimulus | 0.042   | 0.412|
| 2   | Uncharacterized               | E1BEW4               | Regulation of cellular component organization | 0.014   | 0.421|
| 3   | OCRL protein                  | A7E337               | Cellular component assembly; regulation of metabolic process | 0.037   | 0.425|
| 4   | DTYMK protein                 | A5P1V9               | Metabolic process; biosynthetic process; | 0.034   | 0.497|
| 5   | Myosin-7                      | Q9BE39               | Biosynthetic process; metabolic process; | 0.032   | 0.409|
| 6   | Uncharacterized protein       | E1BF95               | Regulation of kinase activity; regulation of metabolic process | 0.048   | 0.420|
| 7   | Mesoderm induction early response protein 2 | A5P1X4 | Regulation of primary metabolic process; regulation of cellular biosynthetic process | 0.037   | 0.466|
| 8   | Uncharacterized protein       | E1BKT3               | Macromolecule localization; intracellular transport | 0.037   | 0.477|
| 9   | Selenoprotein P               | P49907               | Signal transduction; G-protein coupled receptor signaling pathway | 0.042   | 0.480|
| 10  | ERGIC and golgi 2             | Q0U1                 | – | 0.044   | 0.483|
| 11  | Uncharacterized protein       | G5ESP7               | – | 0.012   | 0.516|
| 12  | N-acylglucosamine 2-epimerase | G3MZ53               | Small molecule metabolic process | 0.006   | 0.524|
| 13  | Serine protease HTRA1         | F1NI52               | Regulation of metabolic process; Signal transduction | 0.002   | 0.527|
| 14  | GTP-binding protein SAR1a     | Q3T0D7               | Macromolecule localization; cellular component assembly | 0.049   | 0.528|
| 15  | Uncharacterized protein       | F1MDD5               | Developmental process; cell differentiation | 0.007   | 0.529|
| 16  | Clusterin                     | F1MW1                | Cell death; cellular process | 0.002   | 0.533|
| 17  | Rab5 GDP/GTP exchange factor  | O18973               | Regulation of metabolic process; Regulation of transport | 0.022   | 0.541|
| 18  | Calcyphosin                   | Q0MCC0               | Metabolic process; regulation of cellular catalytic process | 0.001   | 0.558|
| 19  | Uncharacterized protein       | F1MS35               | Cellular component assembly; positive regulation of metabolic process | 0.010   | 0.560|
| No. | Protein name                                                | Uniprot Accession no | GO annotation                                                                 | Biological process                                                                 | Cell component                                                                 | Molecular function                                                                 | P value | V/C  |
|-----|------------------------------------------------------------|----------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------|------|
| 20  | Tissue factor pathway inhibitor 2                         | Q7YRQ8              | Regulation of metabolic process; circulatory system development                | Membrane-bounded organelle; endomembrane system                                   | Endopeptidase regulator activity                                                | 0.000  | 0.563 |
| 21  | TATA box-binding protein-associated factor RNA polymerase I subunit D | Q32LB6            | Regulation of metabolic process                                                | Membrane-bounded organelle                                                        | Nucleic acid binding                                                           | 0.031  | 0.566 |
| 22  | Uncharacterized protein                                    | E18DC9             | Digestive tract development; cell differentiation                              | Cytoplasm; endomembrane system                                                    | Binding                                                                       | 0.032  | 0.570 |
| 23  | Uncharacterized protein                                    | F1N2K8             | Single-organism developmental process                                           | Cytoplasmic part                                                                 | Protein binding                                                                | 0.007  | 0.578 |
| 24  | Uncharacterized protein                                    | F1XHSO             | Regulation of cytoskeleton organization                                         | Cytoplasm                                                                        | Protein binding                                                                | 0.001  | 0.579 |
| 25  | Uncharacterized protein                                    | G3N1L7             |                                                                                  |                                                                                  |                                                                                | 0.004  | 0.582 |
| 26  | MHC (BoLA) class II DR-beta chain                         | Q9TTM7             | Antigen processing and presentation                                           | Extracellular matrix; cytoplasm part                                              | Binding                                                                       | 0.033  | 0.582 |
| 27  | Transmembrane protein 106B                                 | Q3ZC25             | Developmental process; Cell morpho-genesis                                      | Membrane; Cytoplasm                                                               | –                                                                             | 0.002  | 0.582 |
| 28  | Uncharacterized protein                                    | E18M92             | Single-multiprocessor organism process                                          | Plasma membrane region; Cytoskeleton                                              | Cytoskeletal protein binding                                                   | 0.041  | 0.588 |
| 29  | Mitochondrial ribonuclease P protein 1                    | Q2K45              | Nucleic acid metabolic process                                                  | Intra cellular organelle; Nucleoplasm                                             | Catalytic activity; Transferase activity                                       | 0.033  | 0.594 |
| 30  | Periplakin                                                  | M5FK8B             | Developmental process; Cell morpho-genesis                                      | Cytoskeleton; Intra cellular part                                                 | Binding                                                                       | 0.004  | 0.595 |
| 31  | Proteasome assembly chaperone 1                           | Q0P5F2             | Cellular component assembly; Organ development                                 | Cytoplasm; Intra cellular organelle                                               | Proteasome binding                                                            | 0.035  | 0.597 |
| 32  | Uncharacterized protein                                    | E18BX6             | Transport                                                                       | Intra cellular; Lysosome intracellular part                                       | Transferase activity                                                           | 0.009  | 0.607 |
| 33  | Glutathione S-transferase                                 | A5PJE0             | Metabolic process                                                               | Membrane                                                                         | Anion binding                                                                 | 0.016  | 0.607 |
| 34  | Receptor-type tyrosine-protein phosphatase F              | A7MBJ4             | Regulation of response to stimulus; cell development                            | Membrane                                                                         | Anion binding                                                                 | 0.025  | 0.608 |
| 35  | Haloacid dehalogenase-like hydrolase domain-containing protein 3 | Q5E9D6            | Small molecule metabolic process                                                | –                                                                                | Hydrolase activity; phosphatase activity                                       | 0.015  | 0.612 |
| 36  | Uncharacterized protein                                    | F1MD7B             |                                                                                  | Intra cellular organelle part; nuclear part                                       | –                                                                             | 0.015  | 0.614 |
| 37  | Alpha-1-antitrypsinase                                     | P3495S             | Regulation of metabolic process; Negative regulation of catalytic activity      | Endoplasmic reticulum; intra cellular organelle                                   | Glycoprotein binding; enzyme binding; enzyme inhibitor activity                | 0.044  | 0.617 |
| 38  | NUP35 protein                                              | A6QZP3             | Transport                                                                       | Membrane                                                                         |                                | 0.010  | 0.619 |
| 39  | Lysosomal alpha-glucosidase                               | Q9MYM4             | Metabolic process                                                               | Intra cellular organelle; extra cellular organelle                               | Hydrolase activity                                                            | 0.025  | 0.625 |
| 40  | Uncharacterized protein                                    | F1MB10             |                                                                                  |                                                                                  |                                                                                | 0.013  | 0.626 |
| No. | Protein name                                      | Uniprot Accession no | GO annotation                                                                 | Biological process                                                                 | Cell component                                      | Molecular function                        | P value  | V/C  |
|-----|--------------------------------------------------|----------------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|------------------------------------------------------|-------------------------------------------|----------|------|
| 41  | Uncharacterized protein                          | E1B31                | Positive regulation of immune system process                                 | Intracellular organelle; membrane                                                  | Enzyme binding                                      | 0.040 | 0.629          |
| 42  | Proteasome subunit alpha type-2                  | Q3T0Y5               | Cellular protein metabolic process; multi-organism process                    | Extracellular vesicle; intracellular organelle                                     | Endopeptidase activity                              | 0.031 | 0.631          |
| 43  | Uncharacterized protein                          | F1MNT4               | Tissue development; regulation of cellular process                            | Extracellular matrix                                                              | Integrin binding                                    | 0.002 | 1.502          |
| 44  | Up-regulator of cell proliferation               | G3X839               | Apoptotic process                                                            | –                                                                                   | –                                                   | 0.011 | 1.504          |
| 45  | Uncharacterized protein                          | F1MPD4               | –                                                                              | –                                                                                   | –                                                   | 0.000 | 1.508          |
| 46  | Uncharacterized protein                          | G3MZ27               | Single-organism process                                                      | Membrane part                                                                      | Trans-membrane signaling receptor activity          | 0.003 | 1.511          |
| 47  | CD44 antigen                                     | F1MHC3               | Cell adhesion                                                                 | Integral component of membrane                                                     | Binding                                              | 0.000 | 1.512          |
| 48  | Uncharacterized protein                          | G3X6B3               | Immune system process                                                        | Membrane                                                                           | Nucleic acid binding                                 | 0.041 | 1.515          |
| 49  | Leucine-rich repeat flightless-interacting protein 2 | E1BBW0             | –                                                                              | Cytoplasm; membrane                                                                | Protein binding                                      | 0.009 | 1.530          |
| 50  | Tyrrosine-protein phosphatase non-receptor type   | A6QQN2               | Apoptotic signaling pathway                                                   | Endoplasmic reticulum                                                              | Phosphatase activity                                 | 0.006 | 1.537          |
| 51  | Uncharacterized protein                          | G5E6P8               | Lipid metabolic process                                                       | Membrane                                                                           | Hydrolase activity                                   | 0.016 | 1.537          |
| 52  | Mitogen-activated protein kinase 7               | A5PKJ4               | Intracellular transport                                                       | Cytoplasm                                                                          | Kinase binding                                       | 0.044 | 1.541          |
| 53  | 78 kDa glucose-regulated protein                 | Q0MCX2               | Regulation of cell migration                                                   | Cytoplasmic vesicle                                                                | Small molecule binding                               | 0.000 | 1.542          |
| 54  | NSL1 protein                                     | A6QQ16               | Primary metabolic process                                                      | Intracellular organelle                                                            | Hydrolase activity                                   | 0.037 | 1.544          |
| 55  | Tryptophan-tRNA ligase, cytoplasmic              | P17248               | Metabolic process                                                             | Intracellular organelle                                                            | Binding                                              | 0.000 | 1.544          |
| 56  | Uncharacterized protein                          | F1MWL1               | Cellular protein metabolic process                                            | Actin cytoskeleton                                                                  | Hydrolase activity                                   | 0.000 | 1.545          |
| 57  | Uncharacterized protein                          | E1B7E1               | Regulation of developmental process                                           | Intracellular organelle                                                            | Protein binding                                      | 0.027 | 1.546          |
| 58  | Band 4.1-like protein 5                          | Q58CU2               | Metabolic process                                                             | Cytoplasm; membrane                                                                | Protein binding                                      | 0.011 | 1.555          |
| 59  | Myosin-1                                         | Q9BE40               | Metabolic process                                                             | Intracellular organelle                                                            | Binding                                              | 0.027 | 1.558          |
| 60  | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 | A6QL95             | Cellular biosynthetic process                                                 | Endoplasmic reticulum                                                              | Catalytic activity                                   | 0.027 | 1.560          |
| 61  | DYSLS protein                                    | A8E641               | Development process                                                           | Cytoplasm                                                                          | Hydrolase activity                                   | 0.000 | 1.560          |
| 62  | MOB kinase activator 3A                          | Q5BD63               | Development process                                                           | Intracellular                                                                       | Cation binding                                       | 0.001 | 1.561          |
| 63  | Store-operated calcium entry-associated regulatory factor | Q08E24             | Regulation of transport                                                       | Intracellular organelle                                                            | –                                                   | 0.041 | 1.565          |
| 64  | Phosphoribosyl pyrophosphate synthase-associated protein 1 | Q08DW2             | Compound metabolic process                                                    | –                                                                                   | Transferase activity                                 | 0.005 | 1.578          |
| 65  | Uncharacterized protein                          | F1MHHS              | Cell-cell adhesion                                                            | Membrane                                                                           | Signaling receptor activity                          | 0.038 | 1.580          |
| No. | Protein name                                      | Uniprot Accession no | GO annotation          | Biological process                | Cell component   | Molecular function | P value | V/C |
|-----|--------------------------------------------------|----------------------|------------------------|----------------------------------|------------------|--------------------|---------|-----|
| 66  | Nuclear splicing regulatory protein 1            | F1MMV5               | Metabolic process      | Nucleus                          | Binding          | 0.002              | 1.583   |
| 67  | SF3B2 protein                                    | A4FV01               | –                      | Intracellular                    | Nucleic acid binding | 0.027              | 1.585   |
| 68  | Guanine nucleotide-binding protein, beta-1 subunit| A7E3V7               | Cellular response to organic substance | Membrane part                  | Hydrolase activity | 0.004              | 1.604   |
| 69  | Transcription factor MaF                         | A7YY73               | Organ development      | Intracellular                    | Nucleic acid binding | 0.001              | 1.610   |
| 70  | Sorting and assembly machinery component 50 homolog | G3MZZ3             | Protein complex biogenesis | Integral component of membrane | –                | 0.001              | 1.611   |
| 71  | Low-density lipoprotein receptor                 | P01131               | Lipid metabolic process | Plasma membrane raft             | –                | 0.003              | 1.612   |
| 72  | MKK3 protein                                     | A4IFH7               | Inflammatory response  | Intracellular                    | Protein kinase activity | 0.002              | 1.619   |
| 73  | Fibroblast growth factor                         | A6QPP3               | Single-organism developmental process | Plasma membrane                | Kinase regulator activity | 0.029              | 1.619   |
| 74  | Uncharacterized protein                          | E1BIU0               | Cellular protein localization | Membrane                      | Binding          | 0.000              | 1.630   |
| 75  | Uncharacterized protein (Fragment)               | E18L89               | Regulation of immune system process | Membrane                      | Cytokine receptor binding | 0.025              | 1.634   |
| 76  | AP-2 complex subunit beta                        | P63009               | Intracellular protein transport | Membrane                      | Transporter activity | 0.035              | 1.639   |
| 77  | Uncharacterized protein                          | F1MPF7               | Developmental process  | Extracellular organelle          | Binding          | 0.001              | 1.653   |
| 78  | Scavenger receptor cysteine-rich type 1 protein M130 | P85521               | Inflammatory response  | Membrane                        | Receptor activity | 0.000              | 1.658   |
| 79  | ALG2 protein                                     | A4FUG6               | Protein metabolic process | Cytoplasmic part              | Protein binding | 0.000              | 1.660   |
| 80  | Collagen alpha-2(XI) chain                       | F1MRP6               | Extracellular region   | Ion binding                     | 0.001              | 1.668   |
| 81  | Uncharacterized protein                          | E1BMF2               | Protein metabolic process | Extracellular region           | Peptidase activity | 0.002              | 1.677   |
| 82  | Uncharacterized protein                          | F6R9F1               | Cellular response to stimulus | Intracellular                   | Binding          | 0.000              | 1.679   |
| 83  | Radial spoke head protein 3 homolog              | A8E4N3               | –                      | –                               | Ion binding       | 0.031              | 1.680   |
| 84  | Cysteine and glycine-rich protein 2              | Q32LE9               | Developmental process  | Intracellular                    | Ion binding       | 0.005              | 1.683   |
| 85  | Arf-GAP domain and FG-repeat-containing protein 1 | Q2TA45               | Developmental process  | Membrane-bounded vesicle        | Enzyme regulator activity | 0.035              | 1.686   |
| 86  | TACC3 protein                                    | A6QL93               | Response to stimulus  | Intracellular organelle         | Binding          | 0.000              | 1.687   |
| 87  | Myristoylated alanine-rich C-kinase substrate    | P12624               | Response to stimulus  | Cytoplasm                       | Protein binding | 0.006              | 1.697   |
| 88  | ER lumen protein-retaining receptor 1            | P33946               | Regulation of transport | Cytoplasmic part              | Peptide binding | 0.005              | 1.705   |
| 89  | Kelch-like protein 9                             | F1MX00               | Metabolic process      | Intracellular                   | Transferase activity | 0.033              | 1.713   |
| 90  | Wiskott-Aldrich syndrome protein family member 2 | A2VDX6               | Cell migration   | Extracellular region part       | Protein binding | 0.006              | 1.717   |
| 91  | Cystolic carboxypeptidase 3                      | G3N121               | Protein metabolic process | Cytoplasm                      | Binding          | 0.034              | 1.717   |
| 92  | Uncharacterized protein                          | F1MQ43               | Regulation of metabolic process | Membrane                      | Enzyme binding   | 0.000              | 1.737   |
| No. | Protein name                                                                 | Uniprot Accession no | GO annotation                                                                 | Biological process         | Cell component                        | Molecular function            | P value | V/C |
|-----|----------------------------------------------------------------------------|----------------------|-------------------------------------------------------------------------------|----------------------------|---------------------------------------|-----------------------------|---------|-----|
| 93  | Collagen alpha-1(IV) chain                                                 | Q7SIB2               | Single-organism process                                                        | Extracellular region       | Protein binding                      | 0.002         | 1.763 |
| 94  | Uncharacterized protein                                                     | E1BG99               | Regulate[Metabolic process]                                                    | –                          | –                                     | 0.000         | 1.852 |
| 95  | Uncharacterized protein                                                     | E1B9F3               | Cellular developmental process                                                 | Cytoplasm                  | Protein binding                      | 0.000         | 1.863 |
| 96  | Uncharacterized protein                                                     | E1B7H4               | Response to stress                                                             | Cytoplasm                  | Protein kinase activity              | 0.002         | 1.885 |
| 97  | Protein phosphatase inhibitor 2                                             | F1MTZ0               | Regulation of signal transduction                                              | Extracellular region       | Protein binding                      | 0.000         | 1.891 |
| 98  | Fibronectin type 3 and ankyrin repeat domains protein 1                    | F1MCR5               | –                                                                              | Intracellular              | –                                     | 0.000         | 1.901 |
| 99  | Calcium/calmodulin-dependent protein kinase I                              | Q08DQ1               | Regulation of metabolic process                                                | Extracellular region       | Protein binding                      | 0.000         | 1.927 |
| 100 | Nucleoredoxin                                                              | A6QLU8               | Regulation of metabolic process                                                | Extracellular              | Nucleoside binding                   | 0.000         | 1.949 |
| 101 | Uncharacterized protein                                                     | F1MX40               | Metabolic process                                                              | Nucleus                    | Nucleoside binding                   | 0.000         | 1.959 |
| 102 | CA(2+)-dependent carbohydrate-binding protein                              | Q9TRL9               | Regulation of developmental process                                           | Nucleus                    | Binding                               | 0.004         | 1.962 |
| 103 | Uncharacterized protein                                                     | F1MJZ0               | Regulation of system process                                                   | Membrane                   | Signaling receptor activity          | 0.001         | 1.970 |
| 104 | PDZ and LIM domain protein 2                                                | Q3T0C8               | Regulation of metabolic process                                                | Cell junction              | Cation binding                       | 0.002         | 1.979 |
| 105 | Uncharacterized protein                                                     | E1B7M1               | Cellular developmental process                                                 | –                         | Exchange factor activity             | 0.022         | 1.998 |
| 106 | Uncharacterized protein                                                     | F1MQI1               | Cellular protein localization                                                  | Intracellular organelle    | Receptor binding                     | 0.021         | 2.085 |
| 107 | Uncharacterized protein                                                     | F1MWFO               | Regulation of protein metabolic process                                        | Cytoplasmic vesicle        | Phospholipid binding                 | 0.007         | 2.243 |
| 108 | NCK adaptor protein 1                                                       | Q1LZB2               | Regulation of signaling                                                       | Cell-cell junction         | Protein kinase inhibitor activity    | 0.001         | 2.252 |
| 109 | Speegren syndrome nuclear autoantigen 1 homolog                            | G3MWY9               | Regulation of cellular process                                                 | Cytoplasm                  | Protein binding                      | 0.000         | 2.254 |
| 110 | Uncharacterized protein                                                     | E1BM72               | Regulate[Primary metabolic process]                                           | –                         | Ion binding                          | 0.001         | 2.294 |
| 111 | Pescadillo homolog                                                          | A7YW40               | Metabolic process                                                             | Nucleus; intracellular     | Nucleic acid binding                 | 0.028         | 2.434 |
| 112 | Transcription elongation factor SPT5                                        | A7YW40               | Metabolic process                                                             | Nucleus; intracellular     | Binding                              | 0.000         | 2.604 |
| 113 | Uncharacterized protein                                                     | F1MHA1               | Biological regulation                                                         | Intracellular part         | Transferase activity                 | 0.000         | 3.021 |
| 114 | Uncharacterized protein                                                     | F1MSV7               | Regulation of secretion                                                       | Intracellular              | Metal ion binding                    | 0.008         | 3.075 |
| 115 | Vesicle-associated membrane protein 3                                       | G3X752               | Regulation of secretion                                                       | Cytoplasm                  | Binding                              | 0.000         | 3.850 |
| 116 | Uncharacterized protein                                                     | F1MUN7               | –                                                                              | –                         | Binding                              | 0.000         |       |
pathway, and autophagy pathway were all involved in the autophagy process of virus infection. The environmental information involved 11 pathways, mainly focusing on the pathways of viral infection and the interaction of signal molecules (Fig. 4C). Among them, p38-Akt signaling pathway, MAPK signaling pathway, Ras signaling pathway and TNF signaling pathway have been proved to be related to virus infection. The annotated proteins in the category of genetic information processing played a role in the synthesis, transport, proteolysis, and splicingosome of cells (Fig. 4D). The annotated proteins in the organismal systems category were related to antigen processing and presentation, NOD-like receptor signaling, Toll-like receptor signaling, complement and coagulation cascades, and Th1 and Th2 cell differentiation pathway groups. These pathways were correlated with the immune response of the host to virus infection (Fig. 4E). The DEPs annotated in the disease category are shown in Fig. 4F. There are ten pathways clustering in infectious diseases, five of which are associated with viral infections.

According to the profiling of DEPs, a relatively large number of proteins were matched with the MAPK signaling pathway, including FGF13, ERK5, and MKK3. The KEGG pathway analysis revealed that MKK3 was involved in 14 pathways, indicating that MKK3 was a key regulatory protein during BPIV3 infection to MDBK cells (Table. 2).

Validation of the selected proteins by real-time quantitative PCR (qRT-PCR)
To verify the DEPs identified by iTRAQ, the transcriptional levels of eight proteins were measured by qRT-PCR. In this study, the eight proteins were randomly selected for qRT-PCR. The four of them upregulated proteins included AP-2, FGF13, myristoylated alanine-rich C-kinase substrate (MARCS), and MKK3 proteins. The other four downregulated proteins included MHC class II (MHCII), glutathione S-transferase (GSTA1), selenium protein P (SepP), and tissue factor pathway inhibitor (TFPI).

As shown in Fig. 5, the expression levels of these genes were consistent with the iTRAQ results. The results of qRT-PCR further verified the reliability of the iTRAQ experiment.

The effect of the p38 MAPK pathway on BPIV3 replication

BPIV3 infection activating the p38 MAPK pathway
The MAPK pathway plays various roles in intracellular signaling network. MKK3 and MKK6 are recognized as upstream kinases of p38. The results of proteomics analysis showed that the MKK3 level was significantly upregulated after BPIV3 infection (Table. 2). Virus infection is considered as an extracellular stimulant that can activate p38 MAPK pathway [22, 23]. It should be investigated...
Fig. 4 Analysis of the KEGG pathway of the differentially expressed proteins. A genetic information processing B Metabolism; C environmental information processing; D cellular processes; E organismal systems; F diseases
whether BPIV3 infection activated the p38 MAPK pathway after MKK3 activation.

The expression of MKK3, p38, and phospho-p38 in BPIV3-infected cells was detected by western blotting assay. Cell samples were collected at 6, 12, and 24 h post BPIV3 infection. Compared to the mock group, the MKK3 expression levels were increased at different infection time points in the infected group. No change was observed in the p38 protein expression level, while the phospho-p38 expression level was significantly higher in the infected group than in the mock group at 12 h and 24 h after BPIV3 infection (Fig. 6). Thus, BPIV3 infection induced MKK3 activation and p38 phosphorylation. The MKK3 expression level was consistent with previous proteomics results, which further verified the reliability of proteomics analysis.

Fig. 5 Real-time RT-PCR analysis of the DEPs in BPIV3-infected cells and controls. MDBK cells were infected with BPIV3 at MOI = 1 or mock-infected. The cells were collected at 24 hpi for real-time RT-PCR to analyze the relative expression of 8 differential expression genes: A AP-2; B FGF13; C MARCS; D MKK3; E GSTA1; F MHCII; G TFPI2; H SepP.
The effect of inhibiting p38 MAPK activation on BPIV3 replication

To investigate whether the activation of the p38 MAPK pathway promotes BPIV3 proliferation, the cells were treated with SB202190, an inhibitor of the p38 MAPK pathway, at 1 h before infection. The MDBK cells were treated with SB202190 at concentrations of 1.25, 5, and 10 μM. Cell samples were collected at 24 h after infection (MOI = 1).

The results are shown in Fig. 7. The BPIV3 infection induced the phosphorylation of p38. After treatment with the inhibitor SB202190, the expression level of p38 was significantly decreased in a dose-dependent manner, indicating that the phosphorylation of p38 was inhibited by SB202190 (Fig. 7A and B). The BPIV3 virus titer decreased by 1.8 log_{10}TCID_{50}/mL after treatment with 10 μM SB202190, indicating that the p38 MAPK pathway participates in the replication of BPIV3 (Fig. 7C). The results showed that SB202190 could inhibit the proliferation of BPIV3. Thus, BPIV3 activated the p38 MAPK signaling pathway that is involved in its replication.

Discussion

iTRAQ LC-MS/MS is a powerful analytical tool for quantitative proteomics analysis that has been widely used in many studies [24–27]. Gray et al. used 2D gel electrophoresis proteomic to investigate in vitro cellular responses during BPIV3 infection [28]. In the present study, we first applied the iTRAQ LC-MS/MS approach to determine the profiles of DEPs in MDBK cells infected with BPIV3 at 24hpi. A total of 116 DEPs were identified at 24 h after infection. On the basis of GO analysis, the DEPs were classified into 19, 11, and 9 categories for biological processes, cellular components, and molecular functions, respectively (Fig. 3). The pathway analysis identified the pathways based on the number of DEPs (Fig. 4). These data could provide a basis for understanding the pathogenic mechanisms of BPIV3 infection.
The results showed that the PI3K-Akt signaling pathway and the MAPK signaling pathway play important roles in the progression of BPIV3 infection. According to the profiles of DEPs in these two signaling pathways, only ITGB3 was downregulated, while the remaining proteins were upregulated. Interestingly enough, the number of matched proteins in the MAPK signaling pathway was relatively large, including FGF13, ERK5, and MKK3. The KEGG pathway analysis further indicated that MKK3 was involved in 14 pathways, which suggested that MKK3 is a key regulatory protein during BPIV3 infection. Previous studies have shown that the MAPK signaling pathway is a target of respiratory viruses, which regulates various stages of the infection process [29, 30].

The MAPK cascade plays various roles in intracellular signaling network pathways. MKK3 and MKK6 are recognized as upstream kinases of p38 that can directly phosphorylate tyrosine and serine/threonine residues to activate p38 [31]. Viral infection is thought to be an extracellular stimulant that activates this pathway. Immunohistochemical detection showed that the phosphorylation level of p-ERK1/p-p38 in the lungs of sheep infected with infectious salmon anemia virus (ISAV) was significantly increased compared to that in healthy sheep [22]. In our proteomics study, the MKK3 level was significantly upregulated at 24 h after BPIV3 infection compared to that in the control group. Therefore, we detected the protein expression level in the p38 MAPK pathway after BPIV3 infection.

First, we investigated whether BPIV3 infection activates the p38 MAPK pathway. The results showed that BPIV3 induced the phosphorylation of p38 after infection. Compared to the control group, the phosphorylated p38 expression was significantly increased after 6 h of BPIV3 infection, demonstrating that BPIV3 could induce the activation of the p38 MAPK pathway in the early stage of infection.

Multiple extracellular stresses activate the MKK3-p38 MAPK cascade, including specific antigens, proinflammatory cytokines, ultraviolet light, heat shock, and other stress responses [32]. In accordance with the results of the mechanism of Coxsackie virus activation of p38 MAPK, MKK3-p38 MAPK was temporarily activated in the early stage of infection [33]. The same results were found in our study, MKK3-p38 MAPK was activated at 6 h post BPIV3 infection. As the BPIV3 infection was gradually prolonged, the phosphorylation of p38 MAPK was more significantly increased at 24 h after infection.

Fig. 7 Inhibition of activation of the p38 pathway inhibits BPIV3 replication. The MDBK cells were treated with SB202190 at 1.25, 5, and 10 μM concentrations. After 1 h, BPIV3-infected cells were inoculated with MOI = 1. The cell samples were collected at 24 h after infection, and the following tests were performed. (A and B) SB202190 impact on p38MAPK phosphorylation. Cell samples were collected 24 h after infection, lysed with cell lysate, and the expression of phospho-p38 and β-actin in the samples was detected by Western-blot; (C)SB202190 impact on Bpiv3 TCID50. The cell supernatant was collected 24 h after infection, and the titer of the virus was detected by TCID50 assay. ** (P < 0.01)
In the late stage of infection, p38 was still continuously activated, which was speculated to be due to the release of proinflammatory cytokines induced by BPIV3 infection. These released proinflammatory cytokines bound to the receptor further enhanced the activation in the p38 MAPK pathway [34, 35].

Many studies have shown that p38 is required for the replication of viruses. The activation of the MAPK pathways by viruses such as stimulates the JNK and p38 MAPK pathways to promote the release of virions [32]. In porcine reproductive and respiratory syndrome virus infection, the virus replication was inhibited after inhibition of the JNK and p38 pathways [36]. The same results were noted in PEDV infection [37]. To detect the role of the p38 MAPK pathway in BPIV3 replication, virus titer was analyzed. We found the inhibitor SB202190 significantly inhibited BPIV3 replication in a dose-dependent manner. It was also found that p38 expression was inhibited after treatment with SB202190. Compared with the untreated group, the virus titer was significantly decreased in the inhibitor treatment cells. These results revealed that the activation of the p38 MAPK pathway facilitated replication of BPIV3.

Conclusion

In this study, DEPs in BPIV3-infected MDBK cells were identified and quantitatively analyzed by iTRAQ and LC-MS-based proteomics analysis. Most of the DEPs were proteins related to inflammatory response, immune response, and lipid metabolism. Although many significantly up- or downregulated proteins and pathways are closely related to the symptoms or pathological responses to BPIV3 infection, further functional investigations are required to understand the pathogenic mechanisms and molecular responses of host cells to BPIV3 infection.

The results of the present study indicated that BPIV3 infection activates the p38 MAPK pathway, which is essential for its replication. Proteomics and western blot analyses showed that BPIV3 infection activated the p38 MAPK signaling pathway. Our future research will focus on which step of virus replication is affected by p38 activation.

Abbreviations

BPIV3: Bovine parainfluenza virus type 3; BRDC: Bovine respiratory disease complex; iTRAQ: Isobaric tags for relative and absolute quantitation analysis; DEPs: Differentially expressed proteins; TGEV: Transmissible gastroenteritis virus; PEDV: Porcine epidemic diarrhea virus; BRSV: Bovine respiratory syncytial virus; DMEM: Dulbecco’s modified Eagle’s medium; FBS: Fetal bovine serum; CPE: Cytopathic effect; GO: Gene ontology; IFA: Indirect immunofluorescent assay; MOI: Multiplicity of infection; IL-13: Interleukin-13; MAPK: Mitogen-activated protein kinase 7; Foxp3: FOXP transcription inhibitory factor; MARCS: Myristoylated alanine-rich C-kinase substrate; MHC: Major histocompatibility complex II; GSTA1: Glutathione S-transferase; SepP: Selenium protein P; TFPI: Tissue factor pathway inhibitor.

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Author contributions

LL and PL performed proteomics experiment, analysed the experimental data, and were major contributors in writing the manuscript. AC performed cell culture and TCID₅₀ examination. HL performed the qRT-PCR examination. ZL performed the western-blot examination. LY reviewed manuscript. XH designed all experiments and analysed the experimental data. All authors read and approved the final manuscript.

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Declarations

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Consent to publication

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Competing interests

The authors declare that there are no conflicts of interest.

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