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Analysis of protein expression changes of the Vero E6 cells infected with classic PEDV strain CV777 by using quantitative proteomic technique

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A B S T R A C T

Recent outbreaks of porcine epidemic diarrhea virus (PEDV) have caused widespread concern. The identification of proteins associated with PEDV infection might provide insight into PEDV pathogenesis and facilitate the development of novel antiviral strategies. We analyzed the differential protein profile of PEDV-infected Vero E6 cells using mass spectrometry and an isobaric tag for relative and absolute quantification. A total of 126 proteins were identified that were differentially expressed between the PEDV-infected and mock-infected groups (P < 0.05, quantitative ratio ≥ 1.2), among which the expression of 58 proteins was up-regulated and that of 68 proteins was down-regulated in the PEDV-infected Vero E6 cells, involving in integrin β2/β3, cystatin-C. The Gene Ontology analysis indicated that the molecular function of the differentially expressed proteins (DEPs) was primarily related to binding and catalytic activity, and that the biological functions in which the DEPs are involved included metabolism, organismal systems, cellular processes, genetic information processing, environmental information processing, and diseases. Among the disease-related functions, certain anti-viral pathways and proteins, such as the RIG-I-like receptor, Rap1, autophagy, mitogen-activated protein kinase, PI3K-Akt and Jak-STAT signaling pathways, and integrin β2/β3 and cystatin-C proteins, represented potential factors in PEDV infection. Our findings provide valuable insight into PEDV-Vero E6 cell interactions.

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1. Introduction

The porcine epidemic diarrhea virus (PEDV) is an enveloped, single-stranded positive-sense RNA virus that causes porcine epidemic diarrhea (PED), an acute and highly contagious enteric disease in pigs. PED is characterized by severe diarrhea, vomiting, dehydration, and a mortality rate of up to 90% in suckling piglets (Pensaert and Deboeck, 1978). PED was first reported in Belgium and the United Kingdom in 1978, and frequent outbreaks have occurred in various Asian countries (Chen et al., 2010). Since 2007, acute PED outbreaks have continually occurred in Thailand, China, and the USA, which have resulted in substantial economic losses (Puranaveja et al., 2009; Li et al., 2012; Chen et al., 2013; Huang et al., 2013; Marthaler et al., 2013; Stevenson et al., 2013; Yang et al., 2013; Chen et al., 2014). The continued outbreaks of PED, despite control efforts, have caused widespread concern.

The PEDV belongs to the genus Alphacoronavirus, in the family Coronaviridae and order Nidovirales (Belouzard et al., 2012). Previous studies have investigated various control measures to protect against PEDV infection, such as vaccines, diagnostic tools, and therapeutic drugs (Sun et al., 2008; Ren et al., 2011; Sun et al., 2012; Zhu et al., 2013; Guo et al., 2013; Kim and Lee, 2013). Various aspects of PEDV infection remain unclear, for example, swine testis (ST) cells expressing porcine aminopeptidase N of PEDV receptor were not susceptible to PEDV infection. African green monkey kidney (Vero) cells are highly susceptible to PEDV infection, and are widely used for the primary isolation and cultivation of PEDV (Pan et al., 2012; Guo et al., 2014). Therefore, Vero lineages are suitable hosts for understanding the mechanisms of PEDV infection.
Proteomics techniques are effective tools for characterizing protein expression profiles, and have been used widely to investigate disease-associated proteins (Hondermarck et al., 2008; Boja et al., 2011; He et al., 2012; Sun et al., 2013). Among current proteomics methods, quantitative high-throughput proteomics approaches are useful for the analysis of infection-associated proteins of pathogens (Linde et al., 2013; Papachristou et al., 2013; Ye et al., 2013; Zeng et al., 2015). In our current study, we used a quantitative proteomics approach based on an iTRAQ tandem mass spectrometry (MS/MS) technique to identify proteins differentially expressed between PEDV-infected and mock-infected Vero E6 cells. The functions of the differentially expressed proteins (DEPs) were analyzed to determine whether they might be associated with PEDV infection. Our findings provide valuable insight into the changes in cellular processes that occur during PEDV infection.

2. Materials and methods

2.1. Virus, cells, and antibody

The CV777 strain of PEDV, kindly provided by Maurice Pensaert at Ghent University (Merelbeke, Belgium), was used in all of our experiments after being adapted to Vero E6 cells, as previously described (Hofmann and Wyler, 1988). The Vero E6 cell-adapted PEDV, the Vero E6 cells, and the monoclonal antibody against the nucleocapsid protein (Np) of PEDV were stored at the Diarrhea-Related Viruses Section, Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences.

2.2. Viral infection of Vero E6 cells

The Vero E6 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (PBS) in 75-cm2 flasks at 37 °C in a 5% CO2 atmosphere. When the cells reached 70–80% confluence, they were inoculated with the PEDV at a multiplicity of infection of 1 in presence of 5 μg/mL trypsin. At 48 h post-infection, the cells began to exhibit cytopathic effects (CPEs) of viral infection, but no cells lysis or shedding had occurred. The cells were washed three times with cold phosphate-buffered saline (PBS, pH 7.4). A 1.5-mL aliquot of lyssin buffer containing 4% SDS, 1 mM DTT, and 150 mM Tris–HCl (pH 8.0) was added to each flask, and the flasks were incubated at 37 °C for 5 min. The cell lysates were collected using a cell scraper, and boiled for 5 min. Three cell lysate replicates were prepared for the PEDV-infected (V1–V3) and mock-infected (C1–C3) Vero E6 cells, and stored at −80 °C.

2.3. Immunoblotting

Western blotting was performed to confirm PEDV infection by detecting the presence of the Np of PEDV in the Vero E6 cells. Aliquots of the cell lysates were subjected to SDS-PAGE on a 12% acrylamide gel, and the protein bands were transferred to a nitrocellulose membrane using a semi-dry transfer device (Bio-Rad, Hercules, CA, USA). The membrane was blocked using 5% (w/v) nonfat dried milk in PBS at 37 °C for 1 h, before incubation in PBS containing the anti-Np monoclonal antibody (1:2000 dilution) at 37 °C for 1 h. After washing three times with 5% Tween 20 in PBS (PBST), the membrane was incubated in PBST containing a horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000 dilution) at 37 °C for 1 h. After washing three times with PBS, the membrane was incubated with enhanced chemiluminescence detection reagents (Biotopped, Beijing, China) at room temperature for 3 min, and the peroxidase-mediated luminescence was digitally captured using the Molecular Imager ChemiDoc XR+ System (Bio-Rad) and the Image Lab software (Bio-Rad). To verify the differential expression of the selected DEPs, equivalent volumes of the cell lysate replicates from the PEDV-infected (V1–V3) and mock-infected (C1–C3) Vero E6 cells were pooled into the V and C samples, respectively, and western blotting was performed as described above, with the following exceptions: a 1:1000 dilution of the polyclonal antibodies anti-β tubulin, anti-integrin-β3, anti-cystatin-C, anti-protein S100-A2, anti-apolipoprotein E4, and anti-centrin from rabbit (Beijing Biosynthesis Biotechnology, Beijing, China) was used as the primary antibody, and a 1:5000 dilution of the HRP-conjugated goat anti-rabbit IgG (Sigma–Aldrich, St. Louis, USA) was used as the secondary antibody.

2.4. Protein digestion and iTRAQ labeling

Protein digestion of the samples was performed according to the FASP procedure described by Wiśniewski et al. (2009). An aliquot of each cell lysate containing 200 μg of protein was combined with 30 μL of STD buffer containing 4% SDS, 100 mM DTT, and 150 mM Tris–HCl (pH 8.0). The detergent, DTT, and other low-molecular-weight components were removed by dilution in UA buffer containing 8 M Urea and 150 mM Tris–HCl (pH 8.0) and repeated ultrafiltration using Microcon (30 kDa) ultrafiltration units. The reduction of cysteine residues was blocked by the addition of 100 μL of 0.05 M iodoacetamide to the UA buffer. The samples were incubated for 20 min in darkness before ultrafiltration. The Microcon filters were washed three times with 100 μL of UA buffer, followed by two washes with 100 μL DS buffer containing 50 mM triethyl ammonium bicarbonate (pH 8.5). The final protein suspensions were digested using 2 μg of trypsin (Promega, Madison, WI, USA) in 40 μL of DS buffer overnight at 37 °C, and the digested peptides were collected as the filtrate. The peptide content was quantified based on absorbance at 280 nm using an extinction coefficient of 1.1 for a 0.1 mg/mL solution. The digested peptide mixture was labeled using the 8-plex iTRAQ reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. Each iTRAQ reagent was dissolved in 70 μL of ethanol, and added to the digested peptide mixture. The samples were labeled as C1-113, C2-114, C3-115, V1-116, V2-117, or V3-118. The samples were multiplexed, and vacuum dried.

2.5. Peptide fractionation with strong cation exchange chromatography (SCCX)

The iTRAQ labeled peptides were fractionated by SCCX using the AKTA Purifier system (GE Healthcare, Waukesha, WI, USA). The dried peptide mixture was reconstituted, and acidified by the addition of 2 mL of buffer A containing 10 mM KH2PO4 in 25% acetonitrile (pH 2.7). The samples were loaded onto a 4.6 mm × 100 mm column packed with Polysulfoethyl (5 μm, 200 Å) chromatography resin (PolyLC, Columbia, Maryland, USA). The peptides were eluted at a flow rate of 1 mL/min using a gradient of 0–10% buffer B containing 500 mM KCl and 10 mM KH2PO4 in 25% acetonitrile (pH 2.7). The gradient elution consisted of 10–20% buffer B for 25 min, 20–45% buffer B for 5 min, and 50–100% buffer B for 5 min. The absorbance of the eluate was monitored at 214 nm, and fractions were collected at 1-min intervals. Thirty fractions were combined into ten pools, and desalted using Empore standard density SPE C18 cartridges (Sigma–Aldrich, St. Louis, MO, USA) with a bed diameter of 7 mm and a volume 3 mL. Each fraction was concentrated by centrifugation in a vacuum, and reconstituted in 40 μL of 0.1% (v/v) trifluoroacetic acid. All samples were stored at −80 °C until the MS analysis was performed.
2.6. Liquid chromatography (LC) MS/MS analysis

The LC–MS/MS experiments were performed using a Q Exactive mass spectrometer coupled to a Proxeon Biosystem Easy nanoLC (Thermo Fisher Scientific, Waltham, MA, USA). Ten microliters of each fraction was injected for nanoLC–MS/MS analysis. The peptide mixture (5 μg) was loaded onto a C18-reversed phase column (15 cm × 75 μm) packed with RP-C18 (5 μm) resin in buffer A containing 0.1% formic acid, and eluted with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 0.25 μL/min for 140 min using the IntelliFlow technology. The eluate underwent electrospray ionization for the MS/MS analysis. The MS/MS instrument was run in the peptide recognition mode, and the spectra were acquired using a data-dependent top-10 method based on the selection of the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. The determination of the target value was based on the predictive automatic gain control, and the dynamic exclusion duration was 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200, and the resolution for the HCD spectra was set to 17,500 at m/z 200. The normalized collision energy was 30 eV, and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%.

2.7. Identification and analysis of proteins

The MS/MS spectra were compared to the Uniprot Cercopitheciae database (107 051 sequences, downloaded November 25, 2013) and a decoy database using the MASCOT search engine, version 2.2 (Matrix Science, London, UK), embedded in the Proteome Discoverer 1.4 software (Thermo Electron, San Jose, CA). The following parameters were used for protein identification: a peptide mass tolerance of 20 ppm; an MS/MS tolerance of 0.1 Da; trypsin digestion; a missed cleavage value of 2; the fixed modifications included carbamidomethyl, iTRAQ8plex(K), and iTRAQ8plex(ODN-term); the variable modification was oxidation; and an FDR value <0.01. Protein quantification was performed using the Proteome Discoverer 1.4 software based on the centroided reporter ion peak intensity. The average quantitative value of each protein in samples C1, C2, and C3 (mock-infection group) was used as the internal reference. The value of the quantitative ratio for each protein relative to the internal reference was calculated, and averaged to obtain the quantitative ratio (V/C) of the proteins identified in the treatment groups (Unwin et al., 2010). A protein was considered to be differentially expressed between the PEDV-infected and mock-infected groups based on the following criteria: the protein had to be present in three replicates of both groups, the difference in the level of the protein between the two groups had to be statistically significant (P<0.05), and the change ratio for the protein had to be ≥1.2 (Yuan et al., 2012). The expression of a protein with a V/C >1.0 was considered to be up-regulated, and those with a V/C <1.0 were considered to be down-regulated. The data were analyzed using a two-tailed, paired Student’s t test. The statistical analysis was performed using the Excel 2007 software (Microsoft, Redmond, WA, USA). The DEPs were annotated using the Blast2GO, version 2.7.0, program (Ashburner et al., 2000; Quevillon et al., 2005; Götze et al., 2008). The DEPs were blasted against the KEGG Genes database (human). The Gene Ontology categories (GOCs) were retrieved, and mapped to pathways in the KEGG database (Kanehisa et al., 2012).
3. Results

3.1. Identification and analysis of proteins

The Vero E6 cells inoculated with PEDV displayed distinct CPEs at 48 h postinoculation, including cell shrinkage, cell fusion, and a rounded cell morphology, but no cell lysis or shedding was observed (Fig. 1A). The immunoblotting analysis confirmed that the Vero E6 cells were PEDV-infected. The band corresponding to the Np of PEDV was detected in samples V1, V2, and V3, whereas none was detected in samples C1, C2, and C3 (Fig. 1B). The identified peptides, identified proteins, quantified proteins, known/uncharacterized proteins, and the GO annotations are shown in Table 1. A total of 3178 proteins, including 15 564 peptides, were identified in the PEDV-infected and mock-infected groups using the iTRAQ-MS/MS approach, among which 3171 (99.78%) were quantified, 1859 (58.50%) were known proteins, and 1319 (41.50%) were uncharacterized/putative proteins. Based on the GOCs, 2061 (64.85%) of the proteins were annotated as biological process, 2495 (78.51%) were annotated as molecular function, and 1917 (60.32%) were annotated as cellular components.

The quantification and significance of the identified proteins are shown in Fig. 2. The changes in the levels of expression between the two groups were analyzed based on statistical significance. Of the 3178 proteins identified, 2496 (78.54%) were not differentially expressed (P>0.05), and 675 (21.24%) were expressed at statistically different levels between the PEDV-infected and mock-infected Vero E6 cells (P<0.05), including 357 proteins (11.23%) with a P-value between 0.01 and 0.05, 227 proteins (7.14%) with a P-value between 0.001 and 0.01, and 91 proteins (2.86%) with a P-value <0.001. The proteins with a P-value <0.001 were also filtered based on whether the V/C or C/V was ≥1.2. Based on these criteria, a total of 126 (3.96%) of the 3178 identified proteins were determined to have been differentially expressed between the PEDV-infected and mock-infected groups (Table 2). Among the 126 DEPs, 46.03% (58/126) were up-regulated, and 53.97% (68/126) down-regulated. The known proteins and uncharacterized/putative proteins accounted for 69.05% (87/126) and 30.95% (39/126) of the DEPs, respectively. The DEP displaying the greatest increase in expression in the PEDV-infected Vero E6 cells was isoform 2 of the ovarian cancer immunoreactive antigen domain-containing 1 protein (1:2.5), and the DEP displaying the greatest decrease in expression in the PEDV-infected Vero E6 cells was cystatin-C (1:2.2).

3.2. GO annotations of the DEPs

The Gene Ontology (GO) database has been widely used for describing protein function in a standardized format. According to their GOs, the 126 DEPs were annotated as cellular component, biological process, or molecular function. The GO annotations are shown in Table 2, and distributions of the GO annotations are shown in Fig. 3. Seventy-eight DEPs were distributed among 16 groups of biological processes (Fig. 3A). The metabolic process (GO:0008152), cellular process (GO:0009987), single-organism process (GO:0044699), and biological regulation (GO:0005007) groups contained the highest proportions of the biological process DEPs. There were more up-regulated proteins in the cellular component organization group (GO:0071840) than down-regulated proteins. Seventy-four DEPs were distributed among eight cellular component groups (Fig. 3B), among which the organelle (GO:0043226) and cell (GO:0005623) groups contained the highest proportions of cellular component DEPs. There were more down-regulated DEPs in the membrane group (GO:0016020) than up-regulated DEPs, and there were more up-regulated DEPs in the macromolecular complex group (GO:0032991) than down-regulated DEPs. Ninety-seven DEPs were distributed among eight molecular function groups (Fig. 3C), among which the binding (GO:0005488) and catalytic activity (GO:0003824) groups contained the greatest proportion of molecular function DEPs.

3.3. KEGG pathway analysis of the DEPs

The kyoto encyclopedia of genes and genomes (KEGG) pathway is a collection of pathway maps that represent molecular interactions and reaction networks in cells. Seventy-five of the 126 DEPs identified were annotated, and mapped to a total of six KEGG pathway categories, which included the metabolism, organismal systems, cellular processes, genetic information processing, environmental information processing, and diseases pathway categories (Fig. 4). The annotations in the metabolism, organismal systems, and diseases pathway categories represented 32, 25, and 36 pathway groups, respectively (Fig. 4A, B, and F).

The annotations in metabolism pathways category included the carbohydrate, energy, lipid, nucleotide, amino acid, glycan biosynthesis, cofactors and vitamins, biosynthesis of other secondary metabolites, and xenobiotics pathway groups (Fig. 4A). The annotations in the organismal systems category included the Toll-like receptor (TLR) signaling (ko04620), RIG-I-like receptor (RLR) signaling (ko04622), and natural killer cell mediated cytotoxicity (ko04650) pathway groups (Fig. 4B), which represent pathways related primarily to the immune response to virus infection. The largest number of DEPs in the cellular process category were mapped to the lysosome (ko04142) pathway group, all ten of which were down-regulated DEPs (Fig. 4C). The annotations in the genetic information processing category included pathway groups related to DNA replication and repair, transcription, translation, and the folding, sorting, and degradation of proteins (Fig. 4D). The annotations in the environmental information processing categories included pathways related to other small molecule biochemistry.
Table 2
The differentially expressed protein lists between PEDV-infected and mock-infected groups.

| No. | Protein name                                      | UniProtKB accession no. | GO annotation                                                                 | Molecular function            | Cellular component | Biological process                                      | P value     | Average V(|c|) |
|-----|--------------------------------------------------|-------------------------|-------------------------------------------------------------------------------|------------------------------|--------------------|----------------------------------------------------------|-------------|---------------|
| 1   | Cystatin-C or Cystatin-3                        | G7PH52                  | enzyme regulator activity                                                     | –                           | –                  | metabolic process; regulation of biological process      | 6.98E−04    | 0.46          |
| 2²  | Osteopontin precursor                            | F7FL5L                  | –                                                                             | nucleotide binding; catalytic activity | cytoplasm; membrane | development; metabolic process; reproduction; cell differentiation | 9.11E−03    | 0.50          |
| 3   | Retinol dehydrogenase 10                        | G7M2K0                  | –                                                                             | –                           | cytoplasm; membrane | –                                                        | 5.22E−03    | 0.59          |
| 4   | Testis cDNA clone                               | Q4R3Z6                  | –                                                                             | –                           | –                  | –                                                        | 1.36E−03    | 0.59          |
| 5   | Overexpressed in colon carcinoma 1 protein      | H9FAZ7                  | –                                                                             | –                           | –                  | –                                                        | 6.48E−04    | 0.61          |
| 6   | Cytochrome b-245 light chain                    | H9F3U1                  | –                                                                             | membrane                    | –                  | –                                                        | 1.11E−02    | 0.62          |
| 7²  | Centrin-2                                        | F7HKU5                  | metal ion binding; nucleotide binding; catalytic activity                    | –                           | –                  | –                                                        | 1.26E−03    | 0.66          |
| 8²  | Kidney-specific cadherin                        | F6VCT3                  | metal ion binding; catalytic activity                                         | membrane                    | –                  | –                                                        | 6.70E−05    | 0.67          |
| 9²  | Putative WD repeat-containing protein 33        | F6SBK9                  | protein binding                                                               | nucleus                      | –                  | –                                                        | 1.36E−03    | 0.67          |
| 10  | Apolipoprotein E4                                | D5G333                  | –                                                                             | extracellular                | –                  | transport; metabolic process                              | 9.85E−03    | 0.68          |
| 11  | Receptor-type tyrosine-protein phosphatase T isoform 1 | H9F9X9              | catalytic activity                                                            | membrane                    | –                  | metabolic process                                          | 3.56E−03    | 0.68          |
| 12² | Putative neutral and basic amino acid transport protein rBAT-like isoform 3 | F7HIT7              | catalytic activity                                                            | –                           | –                  | metabolic process                                          | 8.18E−05    | 0.69          |
| 13² | Mitochondrial ornithine aminotransferase         | F7BGF3                  | catalytic activity                                                            | cytoplasm; mitochondrial     | –                  | metabolic process; cell organization and biogenesis       | 3.51E−02    | 0.69          |
| 14  | Lyssosomal protective protein                    | G7N4N3                  | catalytic activity                                                            | nucleus                      | –                  | metabolic process                                          | 2.85E−05    | 0.70          |
| 15² | Putative low-density lipoprotein receptor-related protein 2 | F7H113              | metal ion binding; protein binding                                            | cytoplasm; endosome; endoplasmic reticulum; Golgi; membrane | –                  | metabolic process; transport; cell proliferation; development | 1.02E−04    | 0.70          |
| 16  | Alpha-adducin isoform                            | H9FPQ1                  | metal ion binding                                                            | –                           | cytoskeleton       | –                                                        | 3.58E−03    | 0.71          |
| 17  | Integrin beta 2                                  | H9Z8N5                  | receptor activity; protein binding                                           | membrane                    | –                  | cell communication; regulation of biological process; response to stimulus; development metabolic process | 1.82E−04    | 0.71          |
| 18  | Dipetidyl peptidase 2 preproprotein              | H9EXB4                  | catalytic activity                                                            | membrane                    | –                  | –                                                        | 4.22E−03    | 0.71          |
| 19² | Estrogen sulfotransferase                        | F6RUQ2                  | catalytic activity                                                            | nucleus; cytoplasm; membrane | –                  | –                                                        | 4.73E−03    | 0.71          |
| 20² | Putative protein EGK,14077                       | F6PJM4                  | –                                                                             | –                           | –                  | –                                                        | 2.34E−03    | 0.72          |
| 21² | Putative legumain                                | F6S082                  | catalytic activity                                                            | cytoplasm; endosome          | –                  | response to stimulus; metabolic process; regulation of biological process; cell death regulation of biological process; response to stimulus | 2.53E−03    | 0.73          |
| 22  | Metallothionein-1E                               | F6PYY1                  | metal ion binding                                                            | nucleus; cytoplasm           | –                  | –                                                        | 4.86E−03    | 0.74          |
| 23  | Trophoblast glycoprotein                         | H9F4Q1                  | protein binding                                                              | membrane                    | –                  | –                                                        | 6.68E−04    | 0.74          |
| 24² | Putative proactivator polypeptide isoform X6      | F7F376                  | –                                                                             | cytoplasm; vacuole           | –                  | metabolic process                                          | 6.40E−03    | 0.74          |
| 25  | Cathepsin D (Predicted)                          | A9L547                  | catalytic activity                                                            | –                           | –                  | –                                                        | 1.33E−04    | 0.75          |
| 26  | Aldehyde dehydrogenase family 1 member L1        | F7HB04                  | catalytic activity                                                            | cytoplasm; mitochondrial     | –                  | metabolic process                                          | 9.07E−03    | 0.75          |
| No. | Protein name                                                                 | UniProtKB accession no. | GO annotation                                                                 | Cellular component               | Biological process                                                                                 | P value     | Average V(C) |
|-----|------------------------------------------------------------------------------|-------------------------|--------------------------------------------------------------------------------|----------------------------------|-----------------------------------------------------------------------------------------------------|-------------|--------------|
| 27  | Carbonic anhydrase-2                                                         | G7MZP3                  | catalytic activity; metal ion binding                                          | –                                | metabolic process                                                                                    | 1.83E-03    | 0.75         |
| 28  | Erythrocyte band 7 integral membrane protein isoform a                       | F7HP19                  | –                                                                               | cytoskeleton; membrane; extracellular extracellular; nucleus; membrane; cytoskeleton; mitochondrion | cell organization and biogenesis                                                                 | 4.40E-05    | 0.75         |
| 29a | Putative laminin subunit beta-1                                              | F7HPY4                  | catalytic activity; motor activity: signal transducer activity; protein binding; structural molecule activity; nucleotide binding | –                                | metabolic process; regulation of biological process; cell communication; response to stimulus; transport; cellular component movement; development; cell differentiation; cell organization and biogenesis | 1.92E-03    | 0.76         |
| 30a | Putative tissue alpha-L-fucosidase                                           | F7HDC0                  | catalytic activity                                                            | –                                | metabolic process                                                                                    | 6.56E-03    | 0.76         |
| 31  | Similar to human bone marrow stromal cell antigen 1                          | I7GP78                  | catalytic activity                                                            | –                                |                                                                                                    | 2.77E-02    | 0.77         |
| 32  | Solute carrier family 17, member 5                                           | A9X190                  | –                                                                               | membrane                         | transport                                                                                          | 8.00E-03    | 0.77         |
| 33  | Galectin                                                                    | G7N359                  | catalytic activity                                                            | –                                | metabolic process                                                                                    | 2.88E-03    | 0.78         |
| 34  | Sulphhydril oxidase 2                                                        | H9F332                  | catalytic activity                                                            | membrane                         | metabolic process                                                                                    | 4.97E-03    | 0.78         |
| 35  | Folate receptor alpha                                                        | F7BP60                  | –                                                                               | –                                |                                                                                                    | 2.98E-03    | 0.78         |
| 36a | Putative protein EGK,10171                                                   | G7N180                  | –                                                                               | –                                |                                                                                                    | 4.60E-03    | 0.79         |
| 37  | Lyosome-associated membrane glycoprotein 2 isoform B                         | F7BC9K                  | catalytic activity                                                            | –                                |                                                                                                    | 1.61E-03    | 0.79         |
| 38  | RNA-binding motif protein 12B                                                | G7MZR8                  | nucleotide binding                                                            | membrane                         | –                                                                                                  | 2.10E-03    | 0.79         |
| 39  | Similar to human synaptobrevin-like 1 (SYBL1)                                | I7G8H0                  | –                                                                               | membrane                         | transport                                                                                          | 8.83E-03    | 0.79         |
| 40a | Putative polyadenylate-binding protein-interacting protein 1 isoform 2       | F7HOR0                  | DNA binding; RNA binding; protein binding                                       | –                                | metabolic process                                                                                    | 1.17E-04    | 0.79         |
| 41a | Putative versican core protein-like isoform 8                               | F7C5T5                  | metal ion binding; protein binding                                            | extracellular                     | cell differentiation; cellular component movement; development metabolic process                    | 5.67E-05    | 0.79         |
| 42a | Putative N-acetylglucosamine-6-sulfatase                                      | G7PIY5                  | catalytic activity                                                            | cytoplasm; vacuole               |                                                                                                    | 1.14E-04    | 0.80         |
| 43  | ACADSB                                                                      | Q5JK6X                  | catalytic activity; nucleotide binding                                         | –                                | metabolic process                                                                                    | 3.47E-02    | 0.80         |
| 44  | Clusterin                                                                    | Q5ISQ2                  | catalytic activity                                                            | –                                | cell death                                                                                         | 2.54E-03    | 0.80         |
| 45  | Cathepsin Z                                                                  | G7NM43                  | catalytic activity                                                            | –                                | metabolic process                                                                                    | 6.90E-03    | 0.80         |
| 46a | Endoplasmic reticulum resident protein 28                                   | F7GIV5                  | catalytic activity                                                            | –                                | transport                                                                                          | 4.49E-02    | 0.80         |
| 47a | Putative transducin-like enhancer protein 4 isoform 12                       | F6PZC8                  | protein binding                                                               | cytoplasm; endoplasmic reticulum; organelle lumen nucleus | metabolic process; regulation of biological process                                                 | 2.22E-03    | 0.81         |
| 48a | T-cell immunoglobulin and mucin domain-containing protein 1                 | F7GB98                  | protein binding                                                               | –                                |                                                                                                    | 4.71E-05    | 0.81         |
| 49a | Putative N-acetylglucosamine-6-sulfatase                                     | G7NQ90                  | catalytic activity                                                            | –                                | metabolic process                                                                                    | 1.58E-02    | 0.81         |
| 50a | Uncharacterized protein                                                      | F7GQ07                  | catalytic activity                                                            | –                                | metabolic process                                                                                    | 3.01E-04    | 0.81         |
| 51a | Putative galactokinase                                                       | F7DF76                  | nucleotide binding; catalytic activity                                         | cytoplasm                         | metabolic process                                                                                    | 4.72E-02    | 0.81         |
Table 2 (Continued)

| No. | Protein name                                                                 | UniProtKB accession no. | GO annotation                                                                 | Molecular function | Cellular component | Biological process                                                                 | P value    | Average V(ε) |
|-----|------------------------------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------|-------------------|-------------------|-------------------------------------------------------------------------------------|------------|---------------|
| 52  | Signal transducer and activator of transcription                            | Q9N145                   | protein binding; DNA binding; signal transducer activity                        | nucleus; cytoplasm; membrane | cell differentiation; development; metabolic process; regulation of biological process; transport; cell communication; response to stimulus; cell proliferation; reproduction | 7.92E−03 | 0.81          |
| 53  | Delta[14]-sterol reductase                                                    | G7PPJ4                   | catalytic activity                                                              | membrane           | metabolic process                                                                 | 3.51E−02 | 0.81          |
| 54  | Putative disabled homolog 2 isomerase X4                                    | F7GRX9                   | protein binding                                                                 | –                  | –                               | 4.59E−03 | 0.81          |
| 55  | Pyridoxal-dependent dehydrogenase isomerase domain-containing protein 1      | F7GW28                   | catalytic activity                                                              | –                  | metabolic process                                                                 | 1.59E−02 | 0.81          |
| 56  | Rho GTPase-activating protein 29                                              | H9FH14                   | metal ion binding                                                               | membrane           | cell communication; regulation of biological process; response to stimulus        | 1.51E−02 | 0.82          |
| 57  | Protein DBB3                                                                  | F7H2H1                   | –                                                                               | membrane; cytoplasm | –                               | 2.25E−03 | 0.82          |
| 58  | DORA reverse strand protein                                                   | G7Q0P8                   | catalytic activity                                                              | –                  | metabolic process                                                                 | 1.01E−02 | 0.82          |
| 59  | Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (Cyclizing) | G7PPY4                   | catalytic activity; nucleotide binding                                          | membrane           | metabolic process                                                                 | 4.96E−03 | 0.82          |
| 60  | Cadherin 6                                                                    | Q5ISM2                   | metal ion binding                                                               | membrane           | –                               | 6.61E−03 | 0.82          |
| 61  | Putative mitochondrial delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase Fatty acid binding protein, heart | F6NTN1                   | catalytic activity; protein binding                                            | cytoplasm; mitochondrial | –                               | 5.83E−03 | 0.82          |
| 62  | G7MI71                                                                       | G7MI71                   | transporter activity                                                            | –                  | transport                       | 2.36E−03 | 0.82          |
| 63  | Putative NADH dehydrogenase 1 or subcomplex subunit 8                        | F7B3T9                   | –                                                                               | cytoplasm; mitochondrial; membrane | –                               | 1.78E−03 | 0.83          |
| 64  | Endoplasmic reticulum resident protein S8                                      | G7NJK7                   | protein binding                                                                 | cytoplasm; endoplasmic reticulum; organelle lumen | –                               | 5.49E−03 | 0.83          |
| 65  | Agrin                                                                        | H9FU64                   | transporter activity; protein binding                                          | extracellular; membrane | transport; cell communication; regulation of biological process; response to stimulus; cell organization and biogenesis | 9.20E−03 | 0.83          |
| 66  | Toll-interacting protein                                                      | F7DRQ6                   | protein binding                                                                 | –                  | transport                       | 2.53E−02 | 0.83          |
| 67  | Putative tetratranspin-3 isomerase X2                                         | F6SR13                   | –                                                                               | membrane           | –                               | 3.37E−03 | 0.83          |
| 68  | Putative protein EGK,14027                                                    | G7MM35                   | enzyme regulator activity; protein binding                                      | cytoplasm; cytoskeleton; membrane nucleus | metabolic process; regulation of biological process | 6.05E−03 | 0.83          |
| 69  | Insone-5’- monophosphate dehydrogenase 78 kDa glucose-regulated protein       | F6VX4                    | nucleotide binding; catalytic activity; protein binding; metal ion binding      | cytoplasm; cytoskeleton; membrane nucleus | metabolic process; cell proliferation; response to stimulus | 3.12E−04 | 1.20          |
| 70  | 78 kDa glucose-regulated protein                                              | F7C3R1                   | catalytic activity; protein binding                                            | cytoplasm; cytoskeleton; membrane nucleus; cytoplasm; endoplasmic reticulum; cell surface; membrane | cell organization and biogenesis; cell communication; regulation of biological process; response to stimulus; metabolic process; development; cell death | 9.49E−05 | 1.20          |
| 71  | Uncharacterized protein                                                       | F6W6U2                   | –                                                                               | –                  | –                               | 1.40E−02 | 1.20          |
| No. | Protein name | UniProtKB accession no. | GO annotation | Cellular component | Biological process | P value | Average V/C |
|-----|--------------|-------------------------|---------------|-------------------|-------------------|---------|-------------|
| 72<sup>a</sup> | Follistatin-related protein 1 | G7MKF2 | metal ion binding; protein binding | – | – | 3.29E−04 | 1.20 |
| 73<sup>a</sup> | Putative retrotransposon-like protein 1 | F7G2J3 | RNA binding; catalytic activity | – | metabolic process | 6.78E−04 | 1.20 |
| 74<sup>a</sup> | Heat shock protein 105 kDa homolog | F6S529 | nucleotide binding | – | – | 6.60E−05 | 1.21 |
| 75<sup>a</sup> | Putative protein midA homolog | F7GTQ7 | – | – | – | 2.96E−03 | 1.21 |
| 76 | Similar to human S-adenosylhomocysteine hydrolase-like 1 | I7GBN2 | catalytic activity | – | metabolic process; transport | 9.34E−03 | 1.21 |
| 77 | Transferrin receptor 1 | F6UX47 | catalytic activity | extracellular; cytoplasm; endosome; membrane; cell surface | metabolic process; cellular homeostasis; cell differentiation; development; regulation of biological process transport | 4.64E−04 | 1.22 |
| 78<sup>a</sup> | Exocyst complex component 68 | F7GZ4R | – | cytoplasm | – | 5.75E−03 | 1.22 |
| 79 | Protein S100-A2 | H9F670 F7E018 | metal ion binding | membrane; cytoplasm | – | 4.75E−02 | 1.22 |
| 80<sup>a</sup> | Ephrin type-A receptor 2 | I2CY26 | nucleotide binding; RNA binding | – | development; cell differentiation; metabolic process; cell communication; regulation of biological process; response to stimulus; cell death; cell organization and biogenesis; cell proliferation; cellular component movement | 3.02E−02 | 1.22 |
| 81<sup>a</sup> | Putative nucleolar RNA helicase 2-like isoform 3 | F6SQP8 | nucleotide binding; RNA binding; catalytic activity | nucleus | – | 1.12E−03 | 1.23 |
| 82<sup>a</sup> | Putative polyadenylate-binding protein 1-like isoform X2 | F7EJ27 | nucleotide binding | – | – | 1.77E−03 | 1.23 |
| 83<sup>a</sup> | Uncharacterized protein | F7CEU8 | nucleotide binding | – | – | 5.52E−03 | 1.23 |
| 84 | Sequestosome-1 isoform 1 | I2CY26 | metal ion binding | – | – | 8.51E−03 | 1.23 |
| 85 | NEDD8 ultimate buster 1 isoform 2 | H9EZG1 | protein binding | – | metabolic process; regulation of biological process; cell differentiation; defense; response to stimulus | 2.32E−02 | 1.23 |
| 86 | Thioredoxin domain-containing protein 9 | F6YEB0 | – | nucleus; cytoplasm; cytoskeleton | – | 3.79E−02 | 1.23 |
| 87 | Four and a half LIM domains protein 2 | F7CXH4 | protein binding; metal ion binding | cytoplasm; ribosome | ribosome | 2.24E−05 | 1.23 |
| 88 | Similar to human DKFZP564M182 | I7G2J1 | RNA binding; structural molecule activity | cytoplasm; ribosome | – | 2.95E−03 | 1.23 |
| 89 | Similar to human hypothetical protein FJ10634 | I7GWM8 | nucleotide binding | – | – | 1.69E−02 | 1.23 |
| 90<sup>a</sup> | Putative hexokinase-2 | F6Y855 | nucleotide binding; catalytic activity | cytoplasm; membrane; mitochondrial cytoplasm; ribosome; cytosol | – | 3.26E−04 | 1.24 |
| 91 | Ribosomal protein L37 | F7FYI2 | RNA binding; structural molecule activity; metal ion binding | cytoplasm; ribosome; cytosol | – | 3.51E−03 | 1.24 |
| 92 | Pyruvate kinase | F7FI39 | metal ion binding; catalytic activity | – | metabolic process | 1.07E−02 | 1.24 |
| No. | Protein name | UniProtKB accession no. | GO annotation | Molecular function | Cellular component | Biological process | P value | Average V/C
|-----|--------------|------------------------|---------------|--------------------|-------------------|-------------------|---------|------------|
| 93  | Protein phosphatase 1B isoform 2                  | H9EM08                | metal ion binding; catalytic activity | –                  | –                 | metabolic process | 1.40E–02 | 1.24       |
| 94  | Glycogen synthase kinase-3 alpha Phosphorin amino transferase | G7PXQ8                | nucleotide binding; catalytic activity | membrane           | –                 | metabolic process | 4.34E–03 | 1.24       |
| 95  | Histone H2A                                        | H9RCA2                | catalytic activity                   | –                  | chromosome; nucleus; membrane | cell organization and biogenesis; metabolic process | 2.02E–03 | 1.25       |
| 96  | EF-hand domain-containing protein D2              | H9RCS3                | metal ion binding                   | –                  | –                 | –                 | 6.88E–04 | 1.25       |
| 98  | Putative ATP-dependent RNA helicase DDX10         | F7BIN5                | nucleotide binding; catalytic activity | –                  | –                 | –                 | 1.20E–04 | 1.25       |
| 99  | Putative pterin-4-alpha-carbinolamine dehydratase  | F7F694                | catalytic activity; protein binding  | nucleus; cytoplasm | –                 | metabolic process; cell organization and biogenesis | 1.85E–02 | 1.26       |
| 100 | Integrin beta 3                                   | F7FC54                | receptor activity; protein binding   | membrane; cell surface | –                 | cell organization and biogenesis; cell communication; regulation of biological process; response to stimulus; development; cellular component movement; cell proliferation; coagulation | 2.60E–04 | 1.26       |
| 101 | Similar to human exocyst complex component 7     | I7GLB8                | –                                  | cytoplasm           | –                 | transport         | 5.30E–03 | 1.27       |
| 102 | Putative leucine-rich repeat flightless-interacting protein 1 | F6S1D2                | –                                  | –                  | –                 | –                 | 1.48E–03 | 1.27       |
| 103 | Uncharacterized protein                           | G7P6G0                | –                                  | –                  | –                 | –                 | 8.80E–04 | 1.28       |
| 104 | Eukaryotic initiation factor 4A-1 isoform 1      | H9FAB5                | nucleotide binding; RNA binding     | cytoplasm           | –                 | metabolic process | 2.51E–03 | 1.29       |
| 105 | Phosphatidylinositol transfer protein beta isoform isofrom 2 | F7G7C8                | catalytic activity                  | –                  | –                 | transport         | 1.70E–02 | 1.29       |
| 106 | Phosphatidylinositol-3,4,5-triphosphate 5-phosphatase 2 | G7NE91                | protein binding                     | –                  | –                 | metabolic process | 4.14E–03 | 1.30       |
| 107 | Putative tripartite motif-containing protein 47 (TRIM47) | F7HFI4                | protein binding; metal ion binding  | intracellular       | –                 | –                 | 2.65E–02 | 1.31       |
| 108 | UDP-N-acetylhexosamine pyrophosphorylase           | F7CUX2                | catalytic activity                  | –                  | –                 | metabolic process | 3.83E–02 | 1.32       |
| 109 | Putative protein EGR_20713                       | F7B9G5                | protein binding                     | –                  | –                 | –                 | 3.42E–03 | 1.32       |
| 110 | Putative 60S ribosomal protein L23a-like          | F7HD49                | nucleotide binding; structural molecule activity | cytoplasm; ribosome cytoplasm; mitochondrion | – | metabolic process | 3.89E–04 | 1.33       |
| 111 | Mitochondrial inner membrane translocase subunit Tim13 | F6R7Z6                | metal ion binding                   | cytoplasm; ribosome cytoplasm; mitochondrion | – | cell organization and biogenesis; transport | 1.98E–04 | 1.33       |
| 112 | Poly [ADP-ribose] polymerase 9 isoform c Serpin B6 | H9ZSE8                | catalytic activity                  | membrane           | –                 | –                 | 3.46E–03 | 1.34       |
| 113 | EH domain-containing protein 4                    | F7BRQ5                | enzyme regulator activity           | extracellular cytoplasm; endoplasmic reticulum; endosome; membrane | – | metabolic process; transport; cell organization and biogenesis | 7.69E–04 | 1.35       |
| 114 | Calponin-like integrin-linked kinase-binding protein | G7PQP7                | protein binding; catalytic activity | –                  | –                 | cell communication; regulation of biological process; response to stimulus; metabolic process | 5.57E–04 | 1.35       |
category included the PI3K-Akt signaling (ko04151), mitogen-activated protein kinase (MAPK) signaling (ko04010), Jak-STAT signaling (ko04630), TNF signaling (ko04668), and cell adhesion molecule (ko04514) pathway groups [Fig. 4E], all of which represented signal transduction and signaling-molecule interactions that have been shown to be associated with virus infection. The annotations in the diseases category included the human T-cell leukemia virus infection, Epstein–Barr virus infection, hepatitis C, hepatitis B, and measles pathway groups [Fig. 4F], all of which are associated with virus infection involving in three down-regulated proteins.

![Fig. 3](image-url) The Gene Ontology (GO) categories of the differentially expressed proteins at level 2. (A) Biological process GO categories; (B) cellular component GO categories; (C) molecular function GO categories.
proteins and one up-regulated protein. Overall, more disease pathway groups were assigned to a single down-regulated DEP than those assigned to up-regulated DEPs. The integrin (β2 and β3 sub-units) protein was annotated to the largest number of pathway groups (28), which included the organismal systems, environmental information processing, cellular processes, and diseases categories.

3.4. Verification of differential expression

The β tubulin as loading control, three down-regulated DEPs cystatin-C, apolipoprotein E4 and centrin-2, two up-regulated DEPs integrin-β3 and protein S100-A2, were selected to verify differential expression between the PEDV-infected and mock-infected Vero E6 cells. The immunoblotting analysis showed that the ratios of these proteins between the PEDV-infected and mock-infected groups were consistent with those obtained using the quantitative proteomics analysis (Fig. 5).

4. Discussion

In our study, PEDV infection significantly alters protein expression in Vero E6 cells. The differentially expressed proteins (DEPs) annotated to virus infection-associated signaling pathways, autophagy, and virus entry-associated proteins were analyzed further to assess their potential roles in PEDV infection. In mammals, the first line of defense against virus infection is the innate immune system. Early antiviral responses are initiated upon the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), resulting in the production of interferons for the innate immune response and the maturation of dendritic cells for establishing acquired immunity (Yokota et al., 2010). The PRRs are grouped into the TLRs, RLRs, and nucleotide-binding-domain receptors. Our results showed that PEDV infection induced the DEPs that participated in six signaling pathways involved in viral infection, including the RLR, Rap1, PI3K-Akt, MAPK, Jak-STAT, and TLR signaling pathways.

The PEDV is an enteric virus that infects the intestinal epithelial cells (IEC) of swine, causing severe diarrhea. Hirata et al. (2007) reported the RIG-I signaling pathway plays an important role in antiviral innate immunity mechanisms in IECs. Sheikh et al. (2013) reported the Rap1A signaling pathway was associated with secretory diarrhea. The Jak-STAT signaling pathway regulates the adaptive and innate mechanisms related to mucosal immunity (Heneghan et al., 2013; Wang et al., 2013). Our results showed that DEPs induced by PEDV infection in Vero E6 cells involved in the RLR, Rap1, and Jak-STAT signaling pathways. It has been reported that the TLR, MAPK, and PI3K-Akt signaling pathways play roles in host cell responses to coronaviruses (Mizutani et al., 2004; Mizutani et al., 2005).
more DEPs were mapped to the autophagy pathway group than any of the other pathway groups. Fifteen DEPs were mapped to the lysosome and phagosome pathways. Of the 15 proteins, 12 (80%) were down-regulated DEPs. Although the autophagy pathway plays an antiviral role in virus-infected cells, the autophagy machinery is exploited by certain viruses for viral evasion and propagation. Our results showed that PEDV infection induced the downregulation of the expression of many autophagy-associated proteins. Therefore, PEDV infection might inhibit autophagy in Vero E6 cells, thus facilitating virus replication. Previous studies have shown that the microtubule-associated protein 1B is a useful biomarker protein for autophagy (Dong and Levine, 2013). We found that the expression of MAP1B was up-regulated 1.37-fold in the PEDV-infected Vero E6 cells. These results suggest that the PEDV induces autophagy. 

Cystatin-C has been shown to reduce the replication of certain viruses, including the poliovirus, rhinovirus, and human coronaviruses OC43 and 229E (Korant et al., 1986; Collins and Grubb, 1991). The cleavage of S protein has been shown to be essential for the induction of cell-to-cell fusion and coronavirus entry into cells (Sturman et al., 1985). Shirato et al. (2011) reported the transmembrane type II serine protease 2 enhanced infection of PEDV in Vero cells by increasing virus release. In our study, the reduced expression of cystatin-C might facilitate PEDV replication and release through the activation of cysteine-associated proteases in Vero E6 cells. Apolipoprotein E4, galectin, clusterin, and transferrin receptor 1 have also been shown to be associated with viral infection (Hishiki et al., 2010; Peng et al., 2011; Martin and Uprichard, 2013; Tripathi et al., 2013), and may therefore function as infection-associated proteins in PEDV-infected Vero E6 cells. Additionally, the decreased in vitro expression of the adherens junction protein, such as cadherin, might be associated with a reduced integrity of PEDV-infected intestinal epithelial cells in vivo.

To the best of our knowledge, our study represents the analysis of the interactions between PEDV and Vero E6 cells using a quantitative proteomics technique. PEDV infection-associated pathways and proteins are described and discussed based on the bioinformatics analysis of the differentially expressed proteins. Our analysis of Vero E6 cell responses to PEDV infection identified relevant targets for subsequent in-depth studies of PEDV pathogenesis, expand the current knowledge base regarding the interaction between the PEDV and the host cell, and provide useful basic information about other coronaviruses. Although the Vero E6 cells are highly susceptible to PEDV infection and facilitate experimental design and performance for proteomics, the Vero E6 cell line is an interferon-deficient cell line and not a pig cell line. So, the detailed functions of these pathways and proteins in PEDV infection require further verification in the actual host cells of PEDV.

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