Label-Free Quantitative Proteomic Analysis of Differentially Expressed Membrane Proteins of Pulmonary Alveolar Macrophages Infected with Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus and Its Attenuated Strain

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Significant differences exist between the highly pathogenic (HP) porcine reproductive and respiratory syndrome virus (PRRSV) and its attenuated pathogenic (AP) strain in the ability to infect host cells. The mechanisms by which different virulent strains invade host cells remain relatively unknown. In this study, pulmonary alveolar macrophages (PAMs) are infected with HP-PRRSV (HuN4) and AP-PRRSV (HuN4-F112) for 24 h, then harvested and subjected to label-free quantitative MS. A total of 2849 proteins are identified, including 95 that are differentially expressed. Among them, 26 proteins are located on the membrane. The most differentially expressed proteins are involved in response to stimulus, metabolic process, and immune system process, which mainly have the function of binding and catalytic activity. Cluster of differentiation CD163, vimentin (VIM), and nmII as well as detected proteins are assessed together by string analysis, which elucidated a potentially different infection mechanism. According to the function annotations, PRRSV with different virulence may mainly differ in immunology, inflammation, immune evasion as well as cell apoptosis. This is the first attempt to explore the differential characteristics between HP-PRRSV and its attenuated PRRSV infected PAMs focusing on membrane proteins which will be of great help to further understand the different infective mechanisms of HP-PRRSV and AP-PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) has been a leading economically significant viral pathogen of swine worldwide for almost 28 years.[1–3] PRRSV, equine arteritis virus, simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus are members of the family Arteriviridae.[4,5] PRRSV is a positive-sense, single-stranded, RNA virus with a full-length genome of 15 kb that has a 5′ cap and a 3′ poly(A) tail.[4,6–8] PRRSV was first reported in the United States in the late 1980s.[9] In 2006, several large-scale, severe outbreaks of highly pathogenic (HP) atypical PRRSV (HP-PRRSV) were reported in China and neighboring Asian countries.[2,3,10] Reported HP-PRRSV morbidity and mortality rates were much higher than previous pandemic PRRSV strains and associated with more severe clinical presentations and higher rectal temperature (>41 °C).[11] The emergence of HP-PRRSV has caused great economic loss to the swine industry in China and made preventing and controlling PRRSV outbreaks difficult. Therefore, elucidating the causes of the greater virulence of PRRSV and the differences between the HP and attenuated pathogenic (AP) strains has become even more important. To this end, several trials have been conducted to identify virulence factors; these studies have resulted in some successes.[12,13] However, changes in virulence and pathogenic mechanisms are difficult to discern. Other than virulence in vivo, many distinctions in the biological aspects of HP-PRRSV and AP-PRRSV have been noted, such as in viral binding and entry into pulmonary alveolar macrophages (PAMs).

PRRSV exhibits highly restricted cell tropism both in vivo and in vitro.[14] The virus can be detected only in well-differentiated macrophages of lungs, lymph nodes, Peyer's patches, spleen, tonsils, and thymus. PAMs are the main target cells of PRRSV.[15] PRRSV can also replicate in vitro in the African
Significance of the study

Membrane proteins (MPs) of PAMs infected by highly proteomic (HP)- and attenuated proteomic (AP)-PRRSV have been elucidated by LC-MS/MS for label-free quantitative proteomics. Ninety-five differentially expressed proteins were identified and characterized. The most significant difference in the biological process between PAMs infected with HP- and AP-PRRSV is the metabolic process. Most different molecular functions were classified as binding and catalytic activities. Cellular component categories showed that 26 differentially expressed proteins were confirmed as MPs based on the annotation of UniProt database, such as RAP2A, VCL (Vinculin), IFITM3 function in cell–cell junctions, ERK signaling pathway, G protein signaling pathways, biotic stimulus, and so on. Among them, VCL is a kind of F-actin-binding protein which is involved in cell-matrix adhesion and cell–cell adhesion in humans. It was demonstrated that over expression of VCL could inhibit the replication of both HP-PRRSV and the attenuated PRRSV in the mRNA level. There were obvious differences in the inhibiting ability for HP-PRRSV and its attenuated strain. This is the first attempt to explore the differential characteristics between HP-PRRSV and its attenuated PRRSV-infected PAMs focusing on MPs which will be of great help to further understand the different infective mechanisms of HP-PRRSV and AP-PRRSV.

2. Experimental Section

2.1. Ethics Statement

The animal study protocols were approved by the Animal Care and Use Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.2. Viruses and Cell Cultures

HP-PRRSV strain vHuN4\textsuperscript{3,10} at a titer of 10\textsuperscript{5} 50% tissue culture infective dose (TCID\textsubscript{50}) mL\textsuperscript{-1} and cell-passaged attenuated virus strain vHuN4-F112 (AP-PRRSV)\textsuperscript{21,22} at a titer of 10\textsuperscript{5} TCID\textsubscript{50} mL\textsuperscript{-1} were stored as viral stocks. Porcine circovirus 2, classical swine fever virus, PRRSV antibody, and antigen-free 15-day-old piglets were used. Animals were sacrificed in accordance with the ethics statement. Lungs were dissected and lavaged with PBS (PBS; Life Technologies, Inc., Gibco/BRL Division, Grand Island, NY, USA) supplemented with 1% penicillin-streptomycin (Gibco/BRL), then centrifuged at 1000 \times g for 5 min, resuspended in PBS, centrifuged, and resuspended in PBS. PAMs were collected in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco/BRL) containing 10% fetal bovine serum (Gibco/BRL)\textsuperscript{23} and incubated in 10 cm dishes (Corning, Inc., Corning, NY, USA) for 12 h at 37 °C in a 5% CO\textsubscript{2} atmosphere.

2.3. Virus Inoculation

After PAMs were washed with PBS three times, dead and non-adherent cells were removed when confluency exceeded 95%. Three dishes were inoculated with vHuN4 and another three with vHuN4-F112 at multiplicity of infection 1. An additional three dishes were inoculated with DMEM (Gibco-BRL) as a blank control. All dishes were incubated at 37 °C in an atmosphere of 5% CO\textsubscript{2}, as described previously.\textsuperscript{[13]} After incubation for 1 h, inocula were discarded and PAMs were washed with PBS three times. Cell monolayers in all dishes were overlaid with RPMI-1640 medium containing 2% fetal bovine serum and incubated at 37 °C in a 5% CO\textsubscript{2} atmosphere for 24 h.

2.4. Extraction of MPs of PAMs

PAMs were digested with 2 mL 0.25% trypsin-ethylenediaminetetraacetic acid solution (Gibco/BRL), collected by gently pipetting, centrifuged at 1000 \times g for 5 min and lysed using the ProteoExtract Transmembrane Protein Extraction Kits (NOVAGEN, EMD Biosciences, Inc., Madison, WI, USA)\textsuperscript{24,25} according to the manufacturer’s instructions. Cells were resuspended in Extraction Buffer 1 and protease inhibitor cocktail, incubated for 10 min at 4 °C with gentle agitation, and centrifuged at 1000 \times g for 5 min at 4 °C. After removing supernatants, pellets were resuspended in 0.2 mL Extraction Buffer 2A and protease inhibition cocktail, incubated for 45 min at room temperature with gentle agitation, and centrifuged at

green monkey kidney cell line MA-104 and its derivatives, MARC-145 and CL-2621, which are considered permissive cell lines for PRRSV.\textsuperscript{16,17} Reportedly, PRRSV targets cellular membranes (MPs) and enters target cells through receptor-mediated endocytosis during viral infection.\textsuperscript{18} Studies have investigated possible mechanisms employed by PRRSV to infect green monkey kidney cell line MA-104 and its derivative attenuated strain vHuN4-F112\textsuperscript{21,22} using targeted mass spectrometric tool to detect and quantify large amounts of proteins.\textsuperscript{20} Compared with quantitative proteomics using stable isotope labeling such as stable isotope labeling by amino acids in cell culture and isobaric tags for relative and absolute quantitation, LFQP detects greater sensitivity, and have been widely used to analyze host cell responses to viral infection. Among them, liquid chromatography-tandem MS (LC-MS/MS) for label-free quantitative proteomics (LFQP) is an important mass spectrometric tool to detect and quantify large amounts of proteins.\textsuperscript{19}
16 000 × g for 15 min at 4 °C. Supernatants were precipitated with 1 mL acetone and centrifuged at 12 000 × g for 10 min. After evaporating to dryness, 150 μL SDT buffer (4% sodium dodecyl sulfate, 100 mm Tris/HCl at pH 7.6, 0.1 m dithiothreitol) was added and mixtures were heated in boiling water for 5 min. After centrifugation, supernatants were collected and quantified with a BCA Protein Assay Kit (Bio-Rad, USA).

2.5. Label-Free Quantitative Proteomics

2.5.1. Protein Digestion

Digestion of protein (250 μg for each sample) was performed according to the FASP (Filter-Aided Sample Preparation) procedure. Briefly, the detergent, DTT and other low-molecular-weight components were removed using 200 μL UA buffer (8 m Urea, 150 mm Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 10 kD) facilitated by centrifugation. Then 100 μL 0.05 m iodoacetamide in UA buffer was added to block reduced cysteine residues and the samples were incubated for 20 min in darkness. The filter was washed with 100 μL UA buffer three times and then 100 μL 25 mm NH₄HCO₃ twice. Finally, the protein suspension was digested with 3 μg trypsin (Promega) in 40 μL 25 mm NH₄HCO₃ overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm using an extinctions coefficient of 1.1 of 0.1% (g L⁻¹) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.²⁶

2.5.2. LC-MS/MS Analysis

The peptide of each sample was desalted on C18 Cartridges (Empore SPE Cartridges C18 (standard density), bed id 7 mm, volume 3 mL, Sigma), then concentrated by vacuum centrifugation and reconstituted in 40 μL of 0.1% (v/v) trifluoroacetic acid. MS experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). Five microgram peptide was loaded onto a C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (2% acetonitrile and 0.1% Formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid) at a flow rate of 250 nL min⁻¹ controlled by IntelliFlow technology over 120 min. MS data was acquired using a data-dependent top ten method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Determination of the target value is based on predictive automatic gain control. Dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70 000 at m/z 200 and resolution for HCD spectra was set to 17 500 at m/z 200. 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%. The instrument was run with peptide recognition mode enabled. MS experiments were performed triply for each sample.

2.5.3. Sequence Database Searching and Data Analysis

The MS data were analyzed using MaxQuant software version 1.3.0.5. MS data were searched against the uniprot Sus scrofa sequence database (including 34 253 sequences downloaded on 12/27/2014). An initial search was set at a precursor mass window of 6 ppm. The search followed an enzymatic cleavage rule of Trypsin/P and allowed maximal two missed cleavage sites and a mass tolerance of 20 ppm for fragment ions. Carbamidomethylation of cysteines was defined as fixed modification, while protein N-terminal acetylation and methionine oxidation were defined as variable modifications for database searching. The cutoff of global false discovery rate for peptide and protein identification was set to 0.01. Label-free quantification was carried out in MaxQuant as previously described. Protein abundance was calculated on the basis of the normalized spectral protein intensity (LFQ intensity).

All statistical analyses were performed using unpaired t-tests. A p-value <0.05 and ratio >2 or <0.5 were considered to indicate significant differences. Gene Ontology (GO) annotation and functional classification of identified proteins was with Blast2GO ver. V2.6.2 with the current public database b2g_aug 12 (www.Blast2go.com). Identified proteins were classified...
using Blast2go steps under default parameters: blast, mapping, and annotation. Protein–protein interaction networks were analyzed using (String software string-db.org/). Confidence view was assigned a score of 0.4, indicating medium confidence.

2.6. Western Blots

Samples of PRRSV-infected and DMEM-inoculated PAMs were lysed at 24 h post infection and protein concentrations were determined. Samples (20 μg) were separated by 12% SDS-PAGE and transferred to 0.22 μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 and incubated overnight at 4 °C with monoclonal antibodies against heat shock protein 70 (HSP70; ab5439; Abcam plc, Cambridge, UK) or KDEL receptor (ab69659; Abcam plc). After washing three times, membranes were incubated at 37 °C for 60 min with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Abcam plc). Detection used chemiluminescence luminal reagents (Pierce Biotechnology, Waltham, MA, USA).

2.7. Expression Vector Construction and Transfection

The porcine vinculin (VCL) were amplified from the cDNA obtained from PAM cells. Restriction enzyme sites were incorporated into the primer sequences to facilitate molecular cloning. PCR products were cloned into the pCAGGS vector to produce the porcine VCL expression vector. For transfection, cells were seeded in 6-well plates (Corning) and transfected at 70–80% confluency with respective constructed plasmids DNA by using Lipo-fectamine 3000 (Life Technologies), according to the manufacturer’s instructions. The HP-HuN4 or HuN4-F112 was infected in MARC-145 cells at 36 h post transfection. Empty vector transfection samples served as controls in the experiment.

2.8. RNA Extraction, cDNA Synthesis, and Real-time RT-PCR

At 48 h post infection, total RNAs of PRRSV-infected cells were extracted using the RNeasy mini kit (Qiagen), the viral RNAs in supernatants were isolated using QIAamp Viral RNA Mini kit (Qiagen), according to the instruction manual. All the isolated RNAs were used as the template for synthesis of first-strand cDNA by RT-PCR using RT primed by oligo (dT)18 primer using the PrimeScript RT Master Mix (Perfect Real Time, TaKaRa), according to the manufacturer’s instructions. Then the cDNA templates were quantified using PRRSV-specific real-time RT-qPCR.[28]

3. Results

3.1. Label-Free LC-MS/MS for Quantitative Analysis of MPs of PAMs Infected with PRRSVs of Different Virulence

A total of 2849 proteins were detected by LFQP and are displayed in a heatmap (Figure 2). Statistical significance was determined using unpaired t-tests. For all tests, a p-value of <0.05 and ratio of >2 or <0.5 was considered to indicate a significant difference. Glyceraldehyde 3-phosphate dehydrogenase was the internal normalization control and the ratio of glyceraldehyde 3-phosphate dehydrogenase between HP and AP-PRRSV was 0.95 ± 0.55. The “only one exists” group indicated that proteins were detected only in one group but not another group due to low expression level (Table 1). Correlation analysis indicated good repeatability of the technology (Figure 3).

A total of 95 differentially expressed proteins were identified (Table 2). A control group was used to exclude false-positive interference. Data from the control group was also used to elucidate functions of target proteins. Differentially expressed proteins between control and HP-PRRSV-infected cells (Con/HP) and the
Table 1. Overview of differentially expressed proteins.

| ISSUE          | QUANTITATIVE DIFFERENCE | "ONLY ONE EXISTS" |
|----------------|-------------------------|-------------------|
| HP-PRRSV versus CONTROL | 256                      | 158               |
| AP-PRRSV versus CONTROL     | 195                      | 90                |
| HP-PRRSV versus AP-PRRSV     | 52                       | 43                |

p < 0.01 and ratio >2 or <0.5 indicated quantitative difference between two groups. The "only one exists" group indicated that proteins were detected three times in one, but not in the other group. HP-PRRSV group means proteins identified in the HP-PRRSV infected PAMs, while AP-PRRSV was proteins detected in the attenuated pathogenic PRRSV infected PAMs. Control was displayed as the mock.

control and AP-PRRSV-infected cells (Con/AP) are in Supporting Information.

3.2. Subcellular and Functional Characterization and Bioinformatics of Differently Expressed Proteins between the HP-PRRSV and AP-PRRSV Groups

To extend the molecular characterization of quantitative differences and only-one-exists groups, UniProt and GO databases were used to characterize information about biological processes (BPs), molecular functions (MF), and cellular components (CC). BPs of H (vH/vA >2, including 41 proteins) and A (vH/vA < 0.5, including 54 proteins) groups are in Figures 4A and B BP. In group H, GO annotations were primarily distributed in response to stimulus (70.7%), metabolic process (68.3%), and immune system process (43.9%). Ratios were 63.0% response to stimulus, 90.7% metabolic process, and 33.3% immune system process in group A. The most significant difference in BP between PAMs infected with HP-PRRSV and AP-PRRSV was seen for metabolic process, which may be the major reason for the large differences among animals challenged with different virulence of PRRSV.

Molecular function categories of H group and A group were shown in Figure 4A,B MF. Most different molecular functions were classified as binding (87.8 and 90.7%) and catalytic activity (31.7 and 44.4%). Binding of H group included enzyme binding (31.7%), nucleic acid binding (31.7%) and protein complex binding (26.8%), while group A mainly involved nucleic acid binding (31.5%), nucleoside phosphate binding (27.8%) and nucleotide binding (27.8%) from the analysis of GO distribution by level 4. Enzyme code distribution suggested there were five transferases (12.2%), one hydrolase (2.4%), one lyase (2.4%), and two ligases (4.9%) detected in group H. While four oxidoreductases (1.9%), five transferases (9.3%), five hydrolases (9.3%), three lyases (5.6%), and three isomerases (5.6%) in group A.

CC categories were illustrated in Figure 4A B CC. Ninety-five detected differentially expressed proteins were annotated and categorized to CCs of macromolecular complex (58.9%), membrane (57.9%), membrane-enclosed lumen (57.9%), and extracellular region (44.2%). Because of technological problems, we were unable to conclude that all the detected proteins were indeed MPs. However, 26 were confirmed based on the annotation of UniProt database and there also existed proteins partially anchored on the membrane or binding with the MPs.

3.3. Validation of Differentially Expressed MPs by Western Blots

To verify the differentially expressed proteins via LC-MS/MS for LFQP, Western blots were conducted for two proteins partially located on membranes. Expression of HSP70 and the KDEL receptor from cell lysates of vHuN4-infected and vHuN4-F112-infected PAMs, and DMEM-inoculated PAMs were tested with antibodies to the proteins. LFQP showed that the ratios between vHuN4-infected and vHuN4-F112-infected PAMs reached 0.67 and 0.51, respectively. Western blots confirmed LFQP results (Figure 5).

3.4. VCL Protein Inhibits Virus Replication

To examine whether the differentially expressed proteins detected affects virus infection, the VCL transient overexpression vector was transfected into MARC-145 cells, followed by the HP HuN4 or its attenuated strain infection. The PRRSV-specific RT-qPCR results showed that VCL protein could inhibit both viruses, especially for HP-HuN4 strain. Compared with the empty vector

![Figure 3](image-url)
Table 2. Statistics analysis of the 95 differentially expressed proteins between HP-PRRSV group and AP-PRRSV.

| No. | Description                                                                 | UniProt accession | #GOs t-TEST (AP/HP-PRRSV) | HP/AP-PRRSV | No. | Description                                                                 | UniProt accession | #GOs t-TEST (AP/HP-PRRSV) | HP/AP-PRRSV |
|-----|-----------------------------------------------------------------------------|-------------------|---------------------------|-------------|-----|-----------------------------------------------------------------------------|-------------------|---------------------------|-------------|
| 1   | nuclear autoantigen sp-100 isoform 4                                       | tr|RC6158|RC6158_PIG | 3     | 7.12 \times 10^{-05} | 0.069 | 49 Ribonuclease kappa                                                      | tr|F1RF3|F1RF3_PIG | 3     | 1.7E-03 | 0.394 |
| 2   | PREDICTED: collectin-12                                                    | tr|K3H4M9|K3H4M9_PIG | 8     | 2.43 \times 10^{-05} | 3.294 | 50 a4 fmr2 family member 4 isoform x1                                     | tr|F1R16|F1R16_PIG | 0     | 2.41E-03 | 0.222 |
| 3   | Enhancer of mRNA-decapping protein 3                                        | tr|F1S1E7|F1S1E7_PIG | 4     | 1.84 \times 10^{-05} | 0.39 | 51 Kinesin light chain 4 isoform x3                                       | tr|F1RRN1|F1RRN1_PIG | 3     | 3.08E-03 | 6.048 |
| 4   | Low quality protein: atp-binding cassette sub-family b member mitochondrial | tr|F1S8R5|F1S8R5_PIG | 16    | 2.09 \times 10^{-04} | 0.182 | 52 Coatamer subunit beta-like protein                                      | tr|I3LAW4|I3LAW4_PIG | 4     | 8.62E-03 | 4.298 |
| 5   | u1 small nuclear ribonucleoprotein 70kda isoform x1                        | tr|I3N8E5|I3N8E5_PIG | 9     | 7.61 \times 10^{-05} | 0.465 | 53 Heat shock factor-binding protein 1                                     | tr|I3L4U4|I3L4U4_PIG | 1     | N/A     | vH, vA– |
| 6   | Adenylosuccinate lyase                                                     | tr|D2KPI8|D2KPI8_PIG | 13   | 3.15 \times 10^{-04} | 0.283 | 54 m-phase-specific plk-1-interacting protein                             | tr|F1S5C4|F1S5C4_PIG | 4     | N/A     | vH, vA– |
| 7   | Acyl-protein thioesterase 1                                                 | tr|F1SRG9|F1SRG9_PIG | 7     | 7.06 \times 10^{-04} | 2.062 | 55 Ras-related protein rap-2b                                               | sp|Q06AU2|RAP2A_PIG | 20     | N/A     | vH, vA– |
| 8   | IFN regulatory factor 3                                                     | sp|F1S46M|RIF3_PIG | 18   | 1.57 \times 10^{-03} | 0.09  | 56 Histone -like                                                            | tr|F2Z5K9|F2Z5K9_PIG | 16     | N/A     | vH, vA– |
| 9   | ADP-ribosylation factor gtpase-activating protein 2 isoform x1             | tr|F1S1B9|F1S1B9_PIG | 4     | 4.03 \times 10^{-03} | 0.357 | 57 Leucine-rich repeat interacting protein-1                                 | tr|M1FV56|M1FV56_PIG | 4     | N/A     | vH, vA– |
| 10  | Isoform cra_b                                                               | tr|Q29194|Q29194_PIG | 0     | 1.11 \times 10^{-03} | 5.683 | 58 DNAJ homolog subfamily b member 2 isoform x1                            | tr|I1S7R9|I1S7R9_PIG | 17     | N/A     | vH, vA– |
| 11  | Hematological and neurological expressed 1-like protein                     | tr|K7KPSJ|K7KPSJ_PIG | 2     | 2.85 \times 10^{-03} | 0.483 | 59 Heart fatty acid-binding protein                                        | sp|Q02772|FAHBP_PIG | 7     | N/A     | vH, vA– |
| 12  | Raftlin- partial                                                            | tr|I3JL8L|I3JL8L_PIG | 16    | 4.13 \times 10^{-03} | 2.554 | 60 E3 ubiquitin-protein ligase trim56                                       | tr|A0A077ETG0|A0A077-ETG0_PIG | 11     | N/A     | vH, vA– |
| 13  | Nucleoprin seh1-like isoform x2                                              | tr|I3LR4O|I3LR4O_PIG | 6     | 1.79 \times 10^{-03} | 0.133 | 61 Programmed cell death protein 5                                         | tr|F1RX2F|F1RX2F_PIG | 6     | N/A     | vH, vA– |
| 14  | Aconitate mitochondrial                                                     | sp|P16276|ACON_PIG | 6     | 3.83 \times 10^{-03} | 0.469 | 62 Heat shock transcription factor 1                                       | tr|F1RSM7|F1RSM7_PIG | 21     | N/A     | vH, vA– |
| 15  | Thiosulfate sulfurtransferase                                               | tr|F1SKL2|F1SKL2_PIG | 8     | 6.82 \times 10^{-03} | 0.305 | 63 N-alpha-acetyltransferase 50 isoform x1                                  | tr|F2Z5Q7|F2Z5Q7_PIG | 9     | N/A     | vH, vA– |
| 16  | Lysc1_pig ame: full = lysozyme c-1 ame: full = -beta-n-Acetylmyrindase c     | sp|P12067|LYSC1_PIG | 9     | 1.64 \times 10^{-04} | 0.434 | 64 Golgin subfamily a member 2                                             | tr|I3LY2|I3LY2_PIG | 4     | N/A     | vH, vA– |
| 17  | n-acylethanolamine-hydrolyzing acid amidase                                 | tr|F1RYU7|F1RYU7_PIG | 5     | 6.94 \times 10^{-03} | 0.455 | 65 TBC1 domain family member 2a                                            | tr|F1SSG2|F1SSG2_PIG | 2     | N/A     | vH, vA– |
| 18  | Dipeptidyl peptidase 2                                                      | tr|I3LU34|I3LU34_PIG | 0     | 3.18 \times 10^{-03} | 0.33  | 66 IFN-induced protein with tetratricopeptiderepeats 2                      | tr|J7F|H8J|H8_PIG | 6     | N/A     | vH, vA– |

(Continued)
| No. | Description                                      | UniProt accession | GOs (AP/HP-PRRSV) | HP/AP-PRRSV | No. | Description                                      | UniProt accession | GOs (AP/HP-PRRSV) | HP/AP-PRRSV |
|-----|--------------------------------------------------|-------------------|-------------------|-------------|-----|--------------------------------------------------|-------------------|-------------------|-------------|
| 19  | Paraspeckle component 1                          | tF1RN28|F1RN28_PIG         | 2.15 × 10⁻⁰³  | 0.76 | 67 | Urudine 5'-monophosphate synthase                 | tI3LVD6|I3LVD6_PIG         | 0           | N/A          | vH,+vA–       |
| 20  | ADP ATP translocase 3                            | spQ6QRN9|ADT3_PIG          | 6.47 × 10⁻⁰³  | 0.167| 68 | Thymocyte nuclear protein 1-like                 | tF156C1|F156C1_PIG         | 0           | N/A          | vH,+vA–       |
| 21  | Peptidy-prolyl cis-trans isomerase b             | tF15OA2|F15OA2_PIG         | 1.56 × 10⁻⁰³  | 0.406| 69 | Ataxin-2 isoform x2                               | tF1RMZ0|F1RMZ0_PIG         | 17          | N/A          | vH,+vA–       |
| 22  | Translation machinery-associated protein 7       | tF2Z5V1|F2Z5V1_PIG         | 1.63 × 10⁻⁰⁴  | 0.29  | 70 | AP-1 complex subunit beta-1 isoform x3           | tF1RF12|F1RF12_PIG         | 4           | N/A          | vH,+vA–       |
| 23  | Nucleolar and coiled-body phosphoprotein 1 isoform x1 | tF18T1|F18T1_PIG         | 4.06 × 10⁻⁰³  | 0.429| 71 | Vinculin isoform x1                               | spP26234|VINC_PIG          | 26          | N/A          | vH,+vA–       |
| 24  | Histidine–tRNA cytoplasmic isoform x1            | tF1RGD9|F1RGD9_PIG         | 1.36 × 10⁻⁰⁴  | 3.55  | 72 | Tetratricopeptide repeat protein 37             | tF1RNV8|F1RNV8_PIG         | 3           | N/A          | vH,+vA–       |
| 25  | Fch domain only protein 2                        | tI3LSA6|I3LSA6_PIG         | 5.69 × 10⁻⁰³  | 0.278| 73 | Isoleucine–tRNA cytoplasmic                      | tF15UF6|F15UF6_PIG         | 9           | N/A          | vH,+vA–       |
| 26  | Denm domain-containing protein 4c                | tF1SNF8|F1SNF8_PIG         | 3.11 × 10⁻⁰³  | 2.55  | 74 | Collagen alpha-1 chain                           | tF1S258|F1S258_PIG         | 8           | N/A          | vH,+vA–       |
| 27  | Polyribonucleotide 5′-hydroxyl-kinase 3clp1       | tF2Z5N4|F2Z5N4_PIG         | 6.99 × 10⁻⁰³  | 0.136| 75 | SH3 domain-containing kinase-binding protein 1 isoform x1 | tK7GMW4|K7GMW4_PIG         | 4           | N/A          | vH,+vA–       |
| 28  | Endophilin-a2 isoform x1                          | tF1S7L8|F1S7L8_PIG         | 3.23 × 10⁻⁰⁴  | 0.477| 76 | Rho gpase-activating protein 25                 | tF1SPM2|F1SPM2_PIG         | 2           | N/A          | vH,+vA–       |
| 29  | Tyrosine-protein phosphatase non-receptor type 2 isoform x1 | tI3LS25|I3LS25_PIG        | 8.58 × 10⁻⁰³  | 0.419| 77 | Signal transducer and activator of transcription 6 | tE1UC5|E1UC5_PIG         | 9           | N/A          | vH,+vA–       |
| 30  | Unconventional myosin-ib                          | tF1S9U2|F1S9U2_PIG         | 5.24 × 10⁻⁰⁴  | 14.095| 78 | Misshapen-like kinase 1 isoform 1                | tF1RFV9|F1RFV9_PIG         | 13          | N/A          | vH,+vA–       |
| 31  | Ubiquitin-fold modifier 1                        | tM3U242|M3U242_PIG        | 4.32 × 10⁻⁰³  | 0.464| 79 | Coiled-coil domain-containing protein 61 isoform x1 | tF1RMB1|F1RMB1_PIG         | 1           | N/A          | vH,+vA–       |
| 32  | Trafficking protein particle complex subunit 8 isoform x2 | tF1SAL9|F1SAL9_PIG        | 1.06 × 10⁻⁰⁵  | 0.076| 80 | Histone-1                                         | tI3LNN2|I3LNN2_PIG         | 7           | N/A          | vH,+vA–       |
| 33  | Histone a ribonucleoprotein complex subunit 4     | tB7Tj11|B7Tj11_PIG        | 1.43 × 10⁻⁰³  | 0.291| 81 | Csa anaphatosis chemotactic receptor             | tI3LUE7|I3LUE7_PIG         | 12          | N/A          | vH,+vA–       |
| 34  | Heterogeneous nuclear ribonucleoprotein u-like protein 2 | tI3LUR1|I3LUR1_PIG        | 1.36 × 10⁻⁰³  | 0.457| 82 | Pyruvate dehydrogenase e1 component subunit mitochondrial | tI3GH5|I3GH5_PIG         | 7           | N/A          | vH,+vA–       |
| 35  | 3-ketoacyl- coenzyme A synthase x1                | tF1RRB7|F1RRB7_PIG        | 3.82 × 10⁻⁰³  | 0.184| 83 | Ribosome biogenesis protein wdr12               | tF1SHE8|F1SHE8_PIG         | 8           | N/A          | vH,+vA–       |
| 36  | Hepatoma derived growth factor-related protein 3  | tF1R8B4|F1R8B4_PIG        | 3.32 × 10⁻⁰³  | 0.423| 84 | Cleavage stimulation factor subunit 2 tau variant isoform x1 | tF1SDF1|F1SDF1_PIG         | 3           | N/A          | vH,+vA–       |

(Continued)
Table 2. Continued.

| No. | Description                                      | UniProt accession | #GOs (AP/HP-PRRSV) | t-TEST (AP/HP-PRRSV) | HP/AP-PRRSV | No. | Description                                      | UniProt accession | #GOs (AP/HP-PRRSV) | t-TEST (AP/HP-PRRSV) | HP/AP-PRRSV |
|-----|--------------------------------------------------|-------------------|-------------------|---------------------|-------------|-----|--------------------------------------------------|-------------------|-------------------|---------------------|-------------|
| 37  | Serine threonine-protein kinase osr1              | sp|Q863J2|OXSR1_PIG           | 12 | 9.51 × 10^{-04} | 0.385 | 85 | Trifunctional enzyme subunit mitochondrial isoform x2 | tr|F1SDN2|F1SDN2_PIG | 9 | N/A | vH–,vA+ |
| 38  | Apoptosis-associated speck-like protein containing a card isoform 1 | tr|K7GQ17|K7GQ17_PIG          | 22 | 5.20 × 10^{-04} | 0.345 | 86 | Arf-gap domain and fg repeat-containing protein 2 isoform x1 | tr|F1RMY4|F1RMY4_PIG  | 5 | N/A | vH–,vA+ |
| 39  | Trafficking protein particle complex subunit 8   | tr|F15AL8|F15AL8_PIG          | 10 | 2.98 × 10^{-03} | 3.623 | 87 | Sam and sh3 domain-containing protein 3 | tr|F1RTH8|F1RTH8_PIG  | 14 | N/A | vH–,vA+ |
| 40  | Squamous cell carcinoma antigen recognized by t-cells isoform x2 | tr|F1RCA7|F1RCA7_PIG          | 8  | 5.52 × 10^{-03} | 0.34  | 88 | Actin-like protein 6a | tr|F1SGC8|F1SGC8_PIG  | 13 | N/A | vH–,vA+ |
| 41  | Enol-δ delta isomerase mitochondrial              | tr|A9×3T3|A9×3T3_PIG          | 7  | 1.17 × 10^{-03} | 0.493 | 89 | TBC1 domain family member 10b | tr|F1RG61|F1RG61_PIG  | 2  | N/A | vH–,vA+ |
| 42  | wd repeat-containing protein 7 isoform x2        | tr|F1S1×8|F1S1×8_PIG          | 1  | 7.90 × 10^{-03} | 2.052 | 90 | Deubiquitinating protein vcp135 | tr|F1RTZ5|F1RTZ5_PIG  | 3  | N/A | vH–,vA+ |
| 43  | Dipeptidyl peptidase 9                           | tr|M3VH83|M3VH83_PIG          | 0  | 6.78 × 10^{-06} | 37.804 | 91 | GNAT complex locus | tr|A5GFU0|A5GFU0_PIG  | 38 | N/A | vH–,vA+ |
| 44  | Glycerol-3-phosphate dehydrogenase 1-like protein | tr|I3LLU0|I3LLU0_PIG          | 20 | 9.36 × 10^{-03} | 0.461 | 92 | Protein red-like | tr|F1RGE2|F1RGE2_PIG  | 9  | N/A | vH–,vA+ |
| 45  | Serine arginine repetitive matrix prote in 2     | tr|I3LCW3|I3LCW3_PIG          | 6  | 2.18 × 10^{-03} | 0.489 | 93 | Signal-induced proliferation-associated protein 1 | tr|F1RK2|F1RK2_PIG   | 17 | N/A | vH–,vA+ |
| 46  | Protein mago nashi homolog                       | tr|F1S766|F1S766_PIG          | 14 | 3.89 × 10^{-03} | 0.487 | 94 | Protein virilizer homolog | tr|F1RYST1|F1RYST1_PIG | 0  | N/A | vH–,vA+ |
| 47  | Camp-dependent protein kinase catalytic subunit alpha isoform x1 | sp|P36887|KAPCA_PIG         | 3  | 1.25 × 10^{-03} | 2.053 | 95 | GDH 6pg endoplasmic bifunctional protein | tr|K9IVK1|K9IVK1_PIG  | 6  | N/A | vH–,vA+ |

UniProt accession and Seq. description are collected by the blast2go (www.blast2go.com). And the names of the proteins are identified by the accession number from UniProt. #GOs means the quantity of GO annotation, and the HP-PRRSV/AP-PRRSV stands for the ratio between the quantity of expression between HP-PRRSV and AP-PRRSV group.
Figure 4. GO categories of differentially expressed proteins between the HP-PRRSV-infected A) and AP-PRRSV-infected B) BP, biological process GO categories; MF, molecular function GO categories; CC, cellular component GO categories.
control, the virus titer of HP-HuN4 dramatically decreased, while viral titer of attenuated strain mildly decreased, as shown in Figure 6.

4. Discussion

In this study, LFQP of MPs of HP-PRRSV-, AP-PRRSV-infected PAMs and the control was performed. Important information about target proteins related to virus infection was obtained. The HP-PRRSV strain vHuN4 and its derivative, the serially cell-passaged attenuated strain vHuN4-F112,[29] revealed different infection mechanisms in PAMs. A total of 2849 proteins were identified among the control, AP-PRRSV-infected, and HP-PRRSV-infected PAMs. Of these, 2400 were detected in all three groups (Figure 7). We focused on 95 differentially expressed proteins of AP-PRRSV- and HP-PRRSV-infected PAMs; among these, 43 were detected in only one group.

4.1. Identification and Potential Function of MPs in Immunology and Inflammation

PRRSV has been a threat to the global pig economy for several years because of its persistent infection, immune escape, and high mortality from inflammation and high fever.[30] The attenuated PRRSV vHuN4-F112 vaccine strain attenuated from HP-PRRSV vHuN4 by serial passages, which is now used in China.[31]

We analyzed MPs to identify factors associated with immunological effects and determine differences between HP-PRRSV and AP-PRRSV. MPs classified based on GO analysis are in Table 3. A heatmap based on the UniProt database was constructed to comprehend the functions and BPs of differently expressed proteins (Figure 8B), which revealed clustering and abundance of the 26 detected proteins that existed confidentially on the membrane based on UniProt database annotation.

We first focused on proteins associated with immunology and inflammation with higher abundance in AP-PRRSV-infected PAMs. PTPN2 (vH/vA = 0.419), a member of the protein tyrosine phosphatase family, functions as signaling molecule that regulates cellular processes related to the Jak-STAT, IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathways; dephosphorylation of nonreceptor kinases including JAK1, JAK3, STAT3, and STAT6; and negative regulation of IL-2-, IL-4, IL-6, and IFN-mediated signaling. PTPN2 also functions in the response to inflammation via NF-κB.[32–34] SIPA1 (H+/A+) is a mitogen-induced GTPase activating protein for Ras-related regulatory proteins. It was related to G-protein-signaling and blood-brain barrier and immune cell transmigration: VCAM01/CD106 (cluster of differentiation) signaling pathways.[35,36] CSAR1 (H-/A+) is the receptor for the chemotactic and inflammatory peptide anaphylatoxin C5a, which stimulates chemotaxis, granule enzyme release, intracellular calcium release, and superoxide anion production, participates in the innate and adaptive immune responses to...
Table 3. Statistics analysis of proteins that existed definitely on the membrane.

| UniProt accession | Description | Cellular component | Molecular function | Biological process | HP/AP-PRRSV |
|-------------------|-------------|--------------------|-------------------|-------------------|-------------|
| tr|F1SIE7|F1SIE7_PIG | Enhancer of mRNA-decapping protein 3 | Cytoplasmic mRNA processing body membrane | RNA binding; identical protein binding | NA | 0.390 |
| tr|F1SR85|F1SR85_PIG | Low quality protein: ATP-binding cassette sub-family b member mitochondrial | Endosome; endoplasmic reticulum; Golgi apparatus; plasma membrane; integral component of mitochondrial outer membrane; extracellular vesicular exosome | ATP binding; heme-transporting ATPase activity; efflux transmembrane transporter activity; heme binding; ATP catabolic process | Porphyrin-containing compound biosynthetic process; brain development; heme transport; skin development; transmembrane transport | 0.182 |
| tr|I7KJP5|I7KJP5_PIG | Hematological and neurological expressed 1-like protein | Cytoplasm; plasma membrane | NA | NA | 0.483 |
| tr|I3LJL8|I3LJL8_PIG | Raftlin- partial | Endosome; plasma membrane; protein complex; membrane raft; extracellular vesicular exosome | Double-stranded RNA binding | Membrane raft assembly; T cell antigen processing and presentation; protein transport into membrane raft; IL-17 production; ds RNA transport; toll-like receptor 3 signaling pathway; growth; response to exogenous dsRNA; T cell receptor signaling pathway; B cell receptor signaling pathway | 2.554 |
| sp|Q6QRN9|ADT3_PIG | ADP ATP translocase 3 | Nucleus; mitochondrial inner membrane presequence translocase complex; integral component of membrane | ATADP antiporter activity; protein binding | Energy reserve metabolic process; protein targeting to mitochondrion; apoptosis process; ADP transport; ATP transport; viral life cycle; cellular protein metabolic process; small molecule metabolic process; active induction of host immune response by virus; regulation of insulin secretion; transmembrane transport | 0.167 |
| tr|F1SOA2|F1SOA2_PIG | Peptidyl-prolyl cis-trans isomerase b | Nucleus; endoplasmic reticulum; membrane; macromolecular complex; extracellular vesicular exosome | Peptidyl-prolyl cis-trans isomerase activity; protein complex binding poly(A) RNA binding | Protein peptidyl-prolyl isomerization; positive regulation of multicellular organism growth; protein stabilization; bone development; chaperone-mediated protein folding | 0.406 |
| tr|I3LSA6|I3LSA6_PIG | eCh domain only protein 2 | Plasma membrane; coated pit; clathrin-coated vesicle | Phosphatidylinositol-specific phospholipase C activity; phospholipase D activity | Membrane invagination; clathrin coat assembly; clathrin-mediated endocytosis; protein localization to plasma membrane | 0.278 |
| tr|I3SNF8|I3SNF8_PIG | Denn domain-containing protein 4c | Cytosol; plasma membrane; retromer complex; insulin-responsive compartment | Rab guanyl-nucleotide exchange factor activity | Positive regulation of Rab GTPase activity; cellular response to insulin stimulus; protein localization to plasma membrane | 2.550 |

(Continued)
| UniProt accession | Description | Molecular function | Cellular component | Biological process | HP/AP-PRRSV |
|-------------------|-------------|--------------------|-------------------|-------------------|-------------|
| tr|F2Z5N4|F2Z5N4_PIG | Polyribonucleotide 5'-hydroxyl-kinaseclp1 | tRNA-intronendonuclease complex; collagen trimer; mRNA cleavage factor complex; integral component of membrane; extracellular vesicle exosome | ATP binding; signaling pattern recognition receptor activity; low-density lipoprotein particle binding; carbohydrate binding; ATP-dependent polydeoxyribonucleotide 5'-hydroxyl-kinase activity; metal ion binding; ATP-dependent polyribonucleotide 5'-hydroxyl-kinase activity; nucleotide binding; protein kinase activity; ATP binding; protein tyrosine phosphatase activity; protein-protein interaction; integrin binding; protein kinase binding; receptor binding; receptor tyrosine kinase binding; negative regulation of cell proliferation; pattern recognition receptors signaling pathway; mRNA polyadenylation; mRNA cleavage; tRNA splicing via endonucleolytic cleavage and ligation; phagocytosis; recognition; immune response; phosphorylation; cerebellar cortex development; targeting of mRNA for destruction involved in RNA interference; siRNA loading onto RISC involved in RNA interference; negative regulation of tumor necrosis factor-mediated signaling pathway; negative regulation of lipid storage; B cell differentiation; T cell differentiation; erythroid cell differentiation; peptidyl-tyrosine dephosphorylation; negative regulation of epidermal growth factor receptor signaling pathway; negative regulation of tyrosine phosphorylation of Stat1 protein; negative regulation of tyrosine phosphorylation of Stat3 protein; negative regulation of tyrosine phosphorylation of Stat5 protein; negative regulation of tyrosine phosphorylation of Stat6 protein; glucose homeostasis; negative regulation of macrophage differentiation; positive regulation of gluconeogenesis; negative regulation of insulin receptor signaling pathway; negative regulation of inflammatory response; negative regulation of T cell receptor signaling pathway; negative regulation of chemotaxis; negative regulation of IFN-gamma-mediated signaling pathway; negative regulation of type I interferon signaling pathway; regulation of hepatocyte growth factor receptor signaling pathway; negative regulation of IL-2-mediated signaling pathway; negative regulation of IL-4-mediated signaling pathway; negative regulation of IL-6-mediated signaling pathway; negative regulation of IL-12-mediated signaling pathway; negative regulation of prostaglandin E2 signaling pathway; negative regulation of platelet-derived growth factor receptor-beta signaling pathway; negative regulation of positive thymic T cell selection; negative regulation of platelet-derived growth factor receptor-beta signaling pathway | 0.136 |
| tr|I3L9Z5|I3L9Z5_PIG | Tyrosine-protein phosphatase non-receptor type 2 isoform x | Nucleus; endoplasmic reticulum; endoplasmic reticulum-golgi intermediate compartment; plasma membrane | ATP binding; signaling pattern recognition receptor activity; low-density lipoprotein particle binding; carbohydrate binding; ATP-dependent polydeoxyribonucleotide 5'-hydroxyl-kinase activity; metal ion binding; ATP-dependent polyribonucleotide 5'-hydroxyl-kinase activity; nucleotide binding; protein kinase activity; ATP binding; protein tyrosine phosphatase activity; protein-protein interaction; integrin binding; protein kinase binding; receptor binding; receptor tyrosine kinase binding; negative regulation of cell proliferation; pattern recognition receptors signaling pathway; mRNA polyadenylation; mRNA cleavage; tRNA splicing via endonucleolytic cleavage and ligation; phagocytosis; recognition; immune response; phosphorylation; cerebellar cortex development; targeting of mRNA for destruction involved in RNA interference; siRNA loading onto RISC involved in RNA interference; negative regulation of tumor necrosis factor-mediated signaling pathway; negative regulation of lipid storage; B cell differentiation; T cell differentiation; erythroid cell differentiation; peptidyl-tyrosine dephosphorylation; negative regulation of epidermal growth factor receptor signaling pathway; negative regulation of tyrosine phosphorylation of Stat1 protein; negative regulation of tyrosine phosphorylation of Stat3 protein; negative regulation of tyrosine phosphorylation of Stat5 protein; negative regulation of tyrosine phosphorylation of Stat6 protein; glucose homeostasis; negative regulation of macrophage differentiation; positive regulation of gluconeogenesis; negative regulation of insulin receptor signaling pathway; negative regulation of inflammatory response; negative regulation of T cell receptor signaling pathway; negative regulation of chemotaxis; negative regulation of IFN-gamma-mediated signaling pathway; negative regulation of type I interferon signaling pathway; regulation of hepatocyte growth factor receptor signaling pathway; negative regulation of IL-2-mediated signaling pathway; negative regulation of IL-4-mediated signaling pathway; negative regulation of IL-6-mediated signaling pathway; negative regulation of IL-12-mediated signaling pathway; negative regulation of prostaglandin E2 signaling pathway; negative regulation of platelet-derived growth factor receptor-beta signaling pathway; negative regulation of positive thymic T cell selection; negative regulation of platelet-derived growth factor receptor-beta signaling pathway; negative regulation of positive thymic T cell selection; negative regulation of platelet-derived growth factor receptor-beta signaling pathway | 0.419 |
| UniProt accession | Description | Cellular component | Molecular function | Biological process | HP/AP-PRRSV |
|-------------------|-------------|--------------------|--------------------|--------------------|-------------|
| tr|F159U2|F159U2_PIG | Unconventional myos in-xb | **Ruffle; cell cortex; membrane; myosin complex; lamellipodium; filamentous actin; filopodium tip; perinuclear region of cytoplasm** | Microfilament motor activity; actin binding; Rho GTPase activator activity; calmodulin binding; ATP binding; ATPase activity; ADP binding; metal ion binding; | Monocyte chemotaxis; ATP catabolic process; Rho protein signal transduction; establishment of cell polarity; positive regulation of Rho GTPase activity; macrophage chemotaxis; lamellipodium morphogenesis | 14.095 |
| tr|F1RRB7|F1RRB7_PIG | 3-ketoacyl peroxisomal isoform x1 | **Peroxisome; membrane** | Palmitoyl-CoA oxidase activity; transferase activity; transferring acyl groups other than amino-acyl groups; | Very long-chain fatty acid metabolic process; fatty acid beta-oxidation; bile acid metabolic process | 0.184 |
| tr|A9 × 3T3A9 × 3T3_PIG | Enoyl- delta isomerase mitochondrial | **Nucleus; mitochondrion; membrane** | Fatty-acyl-CoA binding; receptor binding; isomerase activity | Fatty acid catabolic process | 0.493 |
| tr|3LLU0|3LLU0_PIG | Glycerol-3-phosphate dehydrogenase 1-like protein | **Plasma membrane; glycerol-3-phosphate dehydrogenase complex; extracellular vesicular exosome** | Glycerol-3-phosphate dehydrogenase [NAD+] activity; sodium channel regulator activity; protein homodimerization activity; ion channel binding; NAD binding | Regulation of heart rate; carbohydrate metabolic process; NADH metabolic process; positive regulation of sodium ion transport; negative regulation of peptidyl-serine phosphorylation; glycerol-3-phosphate catabolic process; oxidation-reduction process; regulation of ventricular cardiac muscle cell membrane depolarization; ventricular cardiac muscle cell action potential; negative regulation of protein kinase C signaling; positive regulation of protein localization to cell surface; regulation of sodium ion transmembrane transporter activity | 0.461 |
| sp|P36887|KAPCA_PIG | cAMP-dependent protein kinase catalytic subunit alpha isoform x1 | **Nucleus; mitochondrion; centrosome; plasma membrane; AMP-activated protein kinase complex; neuromuscular junction; extracellular vesicular exosome; sperm midpiece; ciliary base** | cAMP-dependent protein kinase activity; protein serine/threonine/tyrosine kinase activity; ATP binding; protein kinase binding; ubiquitin protein ligase binding; protein kinase A regulatory subunit binding | Mesoderm formation; neural tube closure; peptidyl-serine phosphorylation; peptidyl-threonine phosphorylation; regulation of osteoblast differentiation; protein autophosphorylation; positive regulation of protein export from nucleus; sperm capacitation; regulation of synaptic transmission; regulation of proteasomal protein catabolic process; regulation of protein processing; positive regulation of cell cycle arrest; cellular response to glucose stimulus; cellular response to parathyroid hormone stimulus; negative regulation of smoothened signaling pathway involved in dorsal/ventral neural tube patterning; regulation of tight junction assembly | 2.053 |
| tr|E7EAX3|E7EAX3_PIG | IFN-induced transmembrane protein 3 | **Integral component of membrane; NA** | | Response to biotic stimulus | 2.862 |
| UniProt accession | Description | Cellular component | Molecular function | Biological process | HP/AP-PRRSV |
|-------------------|-------------|--------------------|--------------------|--------------------|-------------|
| **“Only one exists”** | RAS-related protein rap-2b | Cytosol; plasma membrane; midbody membrane raft; recycling endosome membrane; extracellular vesicular exosome | GTPase activity; GTP binding; GDP binding; protein domain specific binding | GTP catabolic process; negative regulation of cell migration; actin cytoskeleton reorganization; positive regulation of protein autophosphorylation; Rap protein signal transduction; cellular protein localization; regulation of JNK cascade; regulation of dendritic morphogenesis; regulation of protein tyrosine kinase activity; platelet aggregation | VA–vH+ |
| tr|F2Z5K9|F2Z5K9_PIG | Histone-like | Nuclear chromosomes; nucleosomes; nucleoplasm; membrane; extracellular vesicular exosome | DNA binding; chromatin binding; protein heterodimerization activity | Negative regulation of transcription from RNA polymerase II promoter; chromatin silencing at rDNA; DNA replication-dependent nucleosome assembly; blood coagulation; gene expression; DNA methylation on cytosine; cellular response to stress; regulation of gene silencing | VA–vH+ |
| tr|M1F56|M1F56_PIG | Leucine-rich repeat interacting protein-1 Ataxin-2 isoform x2 | Cytoplasm; plasma membrane | DNA binding; protein homodimerization activity | NA | VA–vH+ |
| tr|F1RMZ0|F1RMZ0_PIG | Nucleus; trans-Golgi network; polysome; cytoplasmic stress granule; membrane; perinuclear region of cytoplasm | Epidermal growth factor receptor binding; protein C-terminus binding; poly(A) RNA binding | Negative regulation of receptor internalization; cerebellar Purkinje cell differentiation; cytoplasmic mRNA processing body assembly; stress granule assembly; negative regulation of multicellular organism growth; neuron projection morphogenesis; homeostasis of number of cells; neuromuscular process | VA–vH+ |
| sp|P26234|VINC_PIG | Vinculin isoform x1 | Cytosol; plasma membrane; fascia adherens; focal adhesion; actin cytoskeleton; costamere; protein complex; extracellular vesicular exosome | Dystroglycan binding; actin binding; structural molecule activity; beta-catenin binding; alpha-catenin binding; cadherin binding | Morphogenesis of an epithelium; platelet degranulation; movement of cell or subcellular component; muscle contraction; cell-matrix adhesion; lamellipodium assembly; negative regulation of cell migration; adherens junction assembly; protein localization to cell surface; apical junction assembly; platelet aggregation; epithelial cell–cell adhesion | VA–vH+ |
| tr|I3LUE7|I3LUE7_PIG | C5a anaphylatoxin chemotactic receptor | Integral component of membrane; basolateral plasma membrane; apical part of cell | Aminoacyl-tRNA editing activity; isoleucine-tRNA ligase activity; ATP binding | Osteoblast differentiation; isoleucyl-tRNA aminoacylation; regulation of translational fidelity | VA–vH+ |
| tr|F1SU6|F1SU6_PIG | Isoleucine–tRNA cytoplast mic | Cytosol; membrane; extracellular vesicular exosome | Complement component | Neutrophil chemotaxis; response to peptidoglycan; complement component C5a signaling pathway; mRNA transcription from RNA polymerase II promoter; positive regulation of epithelial cell proliferation; defense response to gram-positive bacterium; positive regulation of ERK1 and ERK2 cascade | VA+vH– |
| (Continued) | | | | | | |
Table 3. Continued.

| UniProt accession | Description | Cellular component | Biological process |
|-------------------|-------------|--------------------|-------------------|
| tif1 | TRVY/1FMY1/VC PIC | Actin-like protein 6a | Regulation of vATPase activity, positive regulation of cell proliferation, nuclear activity of cell cycle |
| tif1 | YCF1/SG2/PC | Nucleoside-cytidine, cytosol | Growth of cell cycle |
| tif1 | YK2/FRK2_PIC | Signal-induced associated protein 1 | Regulation of cell cycle |
| tif1 | YK2/FRK2_PIC | Signal-induced associated protein 1 | Regulation of cell cycle |
| tif1 | YK2/FRK2_PIC | Signal-induced associated protein 1 | Regulation of cell cycle |
| tif1 | YK2/FRK2_PIC | Signal-induced associated protein 1 | Regulation of cell cycle |
| tif1 | YK2/FRK2_PIC | Signal-induced associated protein 1 | Regulation of cell cycle |
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suggests that IFITM3 may help inhibit PRRSV entry and promote resistance to HP-PRRSV infection. Peptidyl-prolyl cis-trans isomerase B (PPIB, vH/vA = 0.406) has peptidyl-prolyl cis-trans isomerase activity and is involved in protein folding and protein peptidyl-prolyl isomerization, which can accelerate protein folding.\[58\] PPIB is positively regulated by viral genome replication, is involved with the viral processes of hepatitis C virus (HCV), and interacts with and stimulates the RNA-binding activity of HCV NS5B. PPIB is critical for efficient replication of the HCV genome.\[59\] Compared with the control group, PPIB was downregulated in the HP-PRRSV and AP-PRRSV groups. The abundance of PPIB was lower in the HP-PRRSV group than the AP-PRRSV group, suggesting an association with PRRSV replication.

### 4.2. Potential Different Approaches Used by Both PRRSVs

String network analysis was used to elucidate interactions among differentially expressed proteins. The essential factors and receptors involved in the entry of PRRSV are reportedly CD163 (Q2VL90), nmHC II-A (MYH9, F1SKJ1), sialoadhesin (SN, A7LCJ3), CD151 (F1RYZ1), and vimentin (VIM) (P02543).\[60-65\] We assessed the involvement of these MPs together with proteins detected by string analysis on viral entry. Receptor proteins involved in PRRSV entry are in Figure 9CD163 (WC1), nmHC II-A (MYH9), and VIM were related to a series of pathway-like regions. Detected virus entry-related receptors and guiding pathway-like regions participated in interactions of AP-PRRSV, HP-PRRSV, and a combination of both (Figure. 9).

To better understand the characteristics of pathway-like regions, the GenomRNAi database was analyzed to annotate detected proteins.\[66\] Regions containing proteins with higher abundance in HP-PRRSV-infected PAMs are green and in AP-PRRSV-infected PAMs are red (Figure 9A). A search of the GenomRNAi database determined that linked proteins IK (IK cytokine), WD repeat domain 12, dyskerin pseudouridine synthase 1, and adenylosuccinate lyase (ADSL) in the green region of Figure 9A shared similar annotations and functioned to decrease expression of NF-κB or IL-8, which are related to inflammation.\[64,65\] Hence, further analysis of these linked proteins may help explain differential mechanisms between HP-PRRSV and AP-PRRSV infection. Other proteins in the green-colored region of 9A are reported to influence virus infection. Nucleolar and coiled-body phosphoprotein 1 increases Sindbis virus infection\[68\] and ADSL increases human papilloma virus 16-GFP infection.\[69\] The lower abundance of nucleolar and coiled-body phosphoprotein and ADSL in the HP-PRRSV-infected group may be related to the host immune response repression of PRRSV and may be used by HP-PRRSV during infection to self-upregulate. The red region of Figure 9A contains some linked proteins with some
Protein-protein interaction network determined by String software showing interactions among differentially expressed proteins between two virulent groups with CD163, VIM, and MYH9 added. Red, protein abundance higher in HP-PRRSV than AP-PRRSV; green, protein abundance in HP-PRRSV lower than in AP-PRRSV. Line colors represent type of evidence for association: green, neighborhood; red, fusion; purple, experimental; light blue, database; black, expression; blue, co-occurrence; yellow, text mining. Gene abbreviations are shown. vH, protein abundance in HP-PRRSV; vA protein abundance in AP-PRRSV. vH/vA = 0, detected only in AP-PRRSV; vH/vA = null, detected only in HP-PRRSV.

Information. Uridine monophosphate synthetase decreases HIV-1 infection, while programmed cell death 5 decreases HCV replication, both downregulate NF-κB expression. The lower abundance of these proteins in the HP-PRRSV group suggested that HP-PRRSV escaped inhibition through an unknown mechanism. Myosin-9 (MYH9) appears to function in cytokinesis, cell shape, and specialized functions such as secretion and capping. MYH9 is an important factor for PRRSV infection and interacts with GP5 of PRRSV, although the underlying mechanisms remain unclear because of limited research. As shown by our results, MYH9 was related to VCL in AP-PRRSV and ACTL6 in HP-PRRSV. VCL is an actin filament (F-actin)-binding protein involved in cell–matrix adhesion and cell–cell adhesion in humans, regulation of cell-surface E-cadherin expression, and potentiation of mechanosensing, and may be important in cell morphology and locomotion by promoting binding with actin, alpha-catenin, cadherin, dystroglycan, and ubiquitin protein ligase. In HIV research, transient overexpression of VCL reduced the susceptibility of human cells to infection with HIV-1 and negatively affected paxillin phosphorylation and limited retroviral infection. Just like HIV-1, in our study, it was demonstrated that overexpression of VCL could inhibit the replication of both HP-PRRSV and the attenuated PRRSV in the mRNA level. There were obvious differences in the inhibiting ability for HP-PRRSV and its attenuated strain. ACTL6A, which is an actin-like protein 6A, is involved in transcriptional...
activation and repression of select genes by chromatin remodeling (alteration of DNA-nucleosome topology) and mainly functions in chromatin binding and transcription coactivator activities.[75] We found that MYH9 may regulate AP-PRRSV and HP-PRRSV infection via different pathways. Through Super Pathways annotation (www.genecards.org), VCL and ACTL6A were identified as participants in the IL-3, IL-5, GM-CSF, and TNF-α/NF-κB signaling pathways, where they may help with differential mechanisms of PRRSV infection with different virulence.

Using the GenomRNAi database, some linked proteins were found to be related to viruses or inflammation (Figure 9B). In the green region, GNAS complex locus (GNAS) increased human papilloma virus 16-GFP,[69] and serine/arginine repetitive matrix 2 decreased HCV infection, influenza A replication and viral numbers, and IL-8 expression.[76–78] Small nuclear ribonucleoprotein U1 subunit 70 decreased influenza A replication and viral numbers.[77] IRF-3 decreased infection by HCV, West Nile virus, and Dengue virus.[76,79] In the red region, HSF1 decreased HCV replication,[76] protein kinase CAMP-activated catalytic subunit alpha decreased VSV infection.[79] Signal transducer and activator of transcription 6 decreased IL-8 expression. RAP2A, MINK1, and GNAS are regulatory proteins in the RAS pathway. RAP2A and MINK1 were detected only in the HP-PRRSV group, whereas GNAS was detected only in the AP-PRRSV group, in accordance with a previous report that stimulation of RAS increases the replication ability of HCV by reducing IFN-JAK-STAT pathway activity.[80] We proposed that PRRSV was also related to the RAS pathway, and differed between HP-PRRSV and AP-PRRSV.

CREB-binding protein (CREBBP) is composed of double-stranded RNA-activated transcription factor with IRF-3, and double-stranded RNA-activated transcription factor is activated in many virus-infected cells to promote apoptosis.[81,82] CREBBP may interact with human herpes virus 8 vIRF-1, which could inhibit the binding of CREBBP to IRF-3.[83] Our results showed that IRF-3 abundance was tenfold lower in the HP-PRRSV than the AP-PRRSV group, which we proposed was a protective mechanism of PRRSV to escape from host immunity and ensure survival after viral infection. The ability of HP-PRRSV HuN4 to induce cell apoptosis is stronger than classical PRRSV (CH-1a) in immune organs and lungs of piglets.[84] We concluded that HP-PRRSV might interact with CREBBP and decrease IRF-3 expression to escape from host immunity and cause severe damage to the cell, highlighting a difference from AP-PRRSV.

Notable differences exist during the infection of AP-PRRSV and HP-PRRSV. HP-PRRSV could inhibit host immune function and evade the immune response via unknown mechanism.[85,86] Label-free MS was performed using AP-PRRSV-infected and HP-PRRSV-infected PAMs. This is the first attempt to explore the differential characteristics between HP-PRRSV and its attenuated PRRSV infected PAMs focusing on membrane proteins. By analyzing detected proteins in HP-infected, AP-PRRSV-infected, and control group, proteins related to the immune response or virus replication were identified that may elucidate unique pathways used by different virulent PRRSVs for cell entry, virus replication, and immune escape mechanisms. Researches on these detected proteins will help with the elucidation of the identity, the expression abundance, and significance of them, future study will be focused on functions of these key membrane proteins to deepen our understanding of differential mechanisms between HP-PRRSV and AP-PRRSV infection.

Abbreviations
ADSL, adenosuccinate lyase; AP, attenuated pathogenic; BP, biological process; CC, cellular component; CD, cluster of differentiation; CREBBP, CREB-binding protein; CSFV, classical swine fever virus; GM-CSF, granulocyte-macrophage colony-stimulating factor; GO, gene ontology; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HP, highly pathogenic; LC-MS/MS, liquid chromatography-tandem MS; LFQ, Label-free quantitative proteomicsMF, label-free quantitative proteomicsMF molecular function; MOI, multiplicity of infections; MP, membrane protein; MYH9, myosin 9; PAMs, pulmonary alveolar macrophages; PCV2, porcine circovirus 2; PPBP, peptidyl-prolyl cis-trans isomerase B; PRRSV, porcine reproductive and respiratory syndrome virus; TCIID50, tissue culture infective dose; v-ATPase, vacuolar ATPase; VCL, vinculin; VIM, vimentin; VSV, vesicular stomatitis virus

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare that they have no conflicts of interest associated with this report.

Keywords
attenuated, highly pathogenic, infection, label-free quantitative proteomics, membrane proteins

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