Proteomic alteration of PK-15 cells after infection by porcine circovirus type 2

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Abstract Porcine circovirus type 2 (PCV2) has been identified as the essential causal agent of post-weaning multisystemic wasting syndrome, which has spread worldwide. To discover cellular protein responses of PK-15 cells to PCV2 infection, two-dimensional liquid chromatography–tandem mass spectrometry (MS) coupled with isobaric tags for relative and absolute quantification (iTRAQ) labeling was employed to quantitatively identify the proteins that were differentially expressed in PK-15 from the PCV2-infected group compared to the uninfected control group. A total of 196 cellular proteins in PK-15 that were significantly altered at different time periods post-infection were identified. These differentially expressed proteins were related to the biological processes of binding, cell structure, signal transduction, cell adhesion, etc. and their interactions. Moreover, some of these proteins were further confirmed by Western blot. The high number of differentially expressed proteins identified should be very useful in elucidating the mechanism of replication and pathogenesis of PCV2 in the future.

Keywords Porcine circovirus type 2 · PK-15 cells · Cellular proteins · iTRAQ

Introduction

Porcine circovirus type 2 (PCV2) is an immunosuppressive virus in pigs. It is a small, nonenveloped, single-stranded DNA virus that belongs to the circoviridae family [1]. The virus genome contains two major open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes the replication proteins which are involved in virus replication, and ORF2 encodes the capsid (Cap) protein [2, 3]. PCV2 has been identified as the etiologic agent of the Postweaning Multisystemic Wasting Syndrome (PMWS) [4, 5] that is widely spread in swine farms and represents one of several porcine circovirus associated diseases (PCVAD). PCV2 infection usually accompanies lymphocyte or monocyte depletion and thus further results in immune suppression in the disease [6, 7]. The immunosuppressive disease mainly presents as PMWS, which caused a great economic loss worldwide [8, 9]. However, the immunosuppressive and pathogenic mechanisms have remained unclear in PCV2-infected pigs.

Proteomics analysis is a powerful technology used in a myriad of studies, including those focused on infectious diseases [10, 11]. Isobaric tags for relative and absolute quantification (iTRAQ) combined with multidimensional liquid chromatography (LC) and tandem MS analysis are emerging as a powerful methodology in the search for disease-specific targets [12, 13]. The iTRAQ reagent labels the primary amines on the peptides and thus can theoretically allow the tagging of most tryptic peptides. The multiplexing ability afforded by the iTRAQ reagents, which are available in four to eight different tags, suited the design of our present study.

Although PCVAD causes substantial economic losses, PCV2 pathogenesis is not fully understood. For elucidation of the interaction between host and PCV2, proteome
analysis has been utilized for host cellular responses to virus infection. Ramírez-Boo [14] used two proteomics strategies, 2-DE and 1-DE, followed by (16)O/(18)O peptide labeling, identification, and quantification via MS, leading to the detection of more than 100 differentially expressed proteins during PCV2 infection in an in vivo environment. Additionally, Zhang et al. [15.] identified 34 host-encoded proteins that were altered in PCV2-infected PK-15 cells using two-dimensional gel electrophoresis (2-DE) coupled with MALDI-TOF/TOF, while Fan and colleagues [16] detected 163 proteins that were significantly affected in PCV2-infected PK-15 cells with the SILAC-based approach. The group of Cheng [17] examined PCV2-infected porcine alveolar macrophages (PAMs) using 2-DE, followed by MALDI-TOF/TOF, and identified 21 host-encoded proteins modified by the virus. A quantitative proteomics approach by our group revealed significant alterations in 145 cellular proteins in PCV2 infected PAMs at different time periods post-infection [18].

In the current study, we described quantitative proteomic analysis of a highly permissive PK-15 cell line (cloned by our laboratory) infected with PCV2 using isobaric tags for relative and absolute quantification, combined with multidimensional liquid chromatography and tandem MS analysis. Overall, we detected 196 proteins showing significant alterations in expression at different time periods post-infection. These proteins may serve as potential biomarkers to establish the interactions between PK-15 and PCV2, and provide novel insights into the mechanisms of disease onset.

Materials and methods

Reagents

Tris-base, SDS, and the 2-D Quantification Kit were purchased from GE Healthcare (Piscataway, NJ, USA). Octane and sequencing grade-modified trypsin were obtained from Sigma-Aldrich (St. Louis, MO). The iTRAQ Reagent Kit was acquired from Applied Biosystems (Foster City, CA). Acetonitrile (ACN) was purchased from Fisher Scientific (Pittsburgh, PA), formic acid (FA) from TEDIA (Fairfield, OH), and trichloroacetic acid (TCA), KH₂PO₄, methanol, acetone, HCl, and KCl from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All buffers were prepared with Milli-Q water (Millipore; Billerica, MA).

Cell culture and virus infection

Porcine circovirus type 2 strain, WG09 (GenBank accession no. GQ845027), was isolated from an intensive pig farm in Shanghai, China, in 2009. The virus stock was a fourth-passage cell culture prepared in PK-15 cells with a titer of 10⁶.⁰ TCID₅₀/mL. PK-15 cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10 % fetal bovine serum (GIBCO, Invitrogen Corporation, CA). Cells were seeded in 25-cm² culture flasks (Costar, Corning Incorporated, NY) until 75 % confluence. Next, cells were inoculated with PCV2 WG09 strain at 1 MOI and collected at 12, 24, 48, and 96 h post-inoculation (hpi), respectively. The amount of fetal bovine serum in medium was decreased to 2 %. Uninfected cells served as the mock infection group (Fig. 1). Viral propagation was confirmed via the indirect immunofluorescence assay and Western blot using a monoclonal antibody against PCV2 Cap protein (made in our laboratory).

Protein isolation, digestion, and labeling with iTRAQ reagents

After culture supernatant was being removed, cells were collected using a cell scraper after the addition of 300 µL lysis buffer (7 M urea, 2 M thiourea, 2 % (w/v) CHAPS) containing a complete protease inhibitor cocktail to the flask. Cells were lysed by sonication, the soluble protein fraction harvested by centrifugation at 15,000 × g for 40 min at 4 °C, and the pellet discarded. The protein concentration in the supernatant was determined using the 2-D Quant Kit (GE Healthcare, Piscataway, NJ). Protein (100 µg) from PK-15 cells was precipitated with acetone overnight at -20 °C and dissolved using iTRAQ dissolution buffer. After reduction and alkylation, protein solutions were digested overnight at 37 °C with sequence graded-modified trypsin (Promega) and labeled with iTRAQ tags, as described in the iTRAQ protocol (Applied Biosystems).

![Porcine kidney cells (PK-15)](images)

PCV2 free or infected

12hmock 12hpi 24hmock 24hpi 48hmock 48hpi 96hmock 96hpi

Protein isolation, reduce/block cysteines, digestion with trypsin and labeled with iTRAQ reagents

Sample combination

SCX-LC/MS/MS analysis

Fig. 1 Strategy for isobaric tags for relative and absolute quantification (iTRAQ)-coupled two-dimensional liquid chromatography–tandem mass spectrometry (2D LC–MS/MS) analysis of PK-15 cells infected with PCV2

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Labeled digests were mixed and dried using a rotary vacuum concentrator (Christ RVC 2-25; Osterode am Harz, Germany). Two independent biological replicates were prepared and analyzed using iTRAQ-based LC–MS/MS.

Off-line 2D LC–MS/MS

The combined peptide mixtures were fractionated via strong cation exchange (SCX) chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu; Kyoto, Japan) using a polysulfoethyl column (2.1 × 100 mm, 5 μm, 200 Å, Poly LC, Columbia, MD). Peptides were eluted with a linear gradient of 0–500 mM KCl (10 mM KH₂PO₄ in 25 % v/v acetonitrile, pH 2.6) for 60 min at a flow rate of 200 μL/min. In total, twenty fractions were collected.

Each fraction was dried, dissolved in 0.1 % FA (formic acid) aqueous solution, and analyzed on a QSTAR XL system (Applied Biosystems, China) interfaced with a 20AD HPLC system (Shimadzu, Kyoto, Japan). Peptides were separated on a reverse-phase Zorbax 300SB-C18 column (75 × 150 mm, 3 μm, 100 Å, Microm, Auburn, CA). The mobile phase was composed of 0.5 % formic acid in water (A) and acetonitrile (B). The flow rate was 400 nL/min with a gradient from 5 % to 45 % B over 70 min and 90 % B over 10 min. MS data were acquired from 400 to 1800, with up to 4 precursors selected for MS/MS from m/z 100 to 2000. Curtain gas was set at 10, nitrogen was used as the collision gas, and the ionization tip voltage was 4000 V.

Data analysis

Relative quantification and protein identification were performed with ProteinPilot™ software (version 3.0, revision 114732, Applied Biosystems) using the Paragon™ algorithm as the search engine. Each MS/MS spectrum was searched against a database of Sus scrofa protein sequences (NCBI nr, released March 2008, downloaded from ftp.ncbi.nih.gov/genomes/Sus_scrofa/protein/). The search parameters allowed for cysteine modification by methyl methanethiosulfonate, and biological modifications were programmed in the algorithm (i.e., amidation, phosphorylation, and semitryptic fragments). All identified proteins required ≥95 % confidence, and the protein confidence threshold cutoff was set to 1.3 (unused) with at least more than one peptide above the 95 % confidence level. The true value for the average ratio was expressed as an error factor (EF = 10 (95 % confidence interval)) and calculated according to the reports. EF <2 was set for satisfactory quantification quality. To designate significant changes in protein expression, fold-changes >1.25 or <0.75 were set as cutoff values. To decrease artificial error, the bias correction option was executed. In addition, one-way analysis of variance (ANOVA) and LSD analysis (SPSS 18.0) were used to determine whether the protein was significantly regulated over time. Differences were considered statistically significant for P values <0.05.

Bioinformatics

Proteins that met the criteria for differential expression were compared with hierarchical cluster analysis using Cluster 3.0 program [19]. Data were displayed using Java Tree View [20]. The molecular functions and subcellular localizations of the unique proteins identified were classified using Protein Center software (DAVID Functional Annotation Tools) [21, 22]. The main annotation types were obtained from the gene ontology consortium Web site (http://david.abcc.ncifcrf.gov/). The protein–protein interaction network was analyzed via STRING software (http://string.embl.de/) [23].

Immunofluorescence assay (IFA)

PCV2 infected cells were washed with PBS, fixed with cold acetone/methanol (1/1 v/v) for 20 min at −20 °C, and allowed to air-dry. Fixed cells were incubated with pig anti-PCV2 polyclonal antiserum (VMRD, USA) at 37 °C for 1 h, washed three times with PBST (0.05 % Tween-20 in PBS, pH 7.4), and further incubated with staphylococcal protein A (SPA) conjugated to FITC (Boshide, Wuhan, China) at 37 °C for 1 h in the dark. After three washes with PBST, infected cells were quantified using Zeiss LSM510 laser confocal microscopy.

Western blot

Samples of PCV2-infected and uninfected PK-15 cells were lysed at 12, 24, 48, and 96 hpi (hours post-infection), and the protein concentrations determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Product No. 23227, USA). Equivalent amounts of cell lysate proteins were subjected to 12 % SDS-PAGE and transferred to 0.22 μm nitrocellulose membranes (Hybond-C extra, Amersham Biosciences). After blotting, membranes were incubated at 37 °C for 60 min, respectively, with mouse monoclonal antibodies (mAbs) to actin (Abcam, Cambridge, UK), vimentin (Santa Cruz Biotechnology, CA), Ras-related protein Rab-11A (Santa Cruz Biotechnology, CA), Hsp90 (Abcam, Cambridge, UK), PCV2 Cap protein (made in our laboratory), or rabbit polyclonal antibody to Annexin I (Santa Cruz Biotechnology, CA). After washing three times with 0.05 % PBST, membranes were incubated.
Confirmation of PCV2 propagation in PK-15 via IFA

Since PCV2 does not induce a typical cytopathic effect (CPE) in PK-15 cells, viral infection was confirmed by detection of PCV2 antigen using IFA at 12, 24, 48, and 96 hpi. The results clearly revealed green fluorescence in PCV2-infected PK-15 cells, which was absent in mock-infected cells. Fluorescence microscopy observations indicated that PCV2 titers increase during the first 24 h of infection, and 70–80 % of cells treated with PCV2 are infected at 96 hpi (Fig. 2).

Protein profile with iTRAQ-coupled 2D LC–MS/MS analysis

Protein extracts were prepared separately from PCV2-infected PK-15 cells at 12, 24, 48, and 96 hpi and virus-free PK-15 cells at the same time points as the mock control group. Overall, 711 proteins were detected using iTRAQ-coupled 2D LC–MS/MS analysis. Among these, 196 proteins displayed significantly altered expression post-infection. As shown in Fig. 3 and Table 1, significantly altered proteins were divided into eight clusters: (1) up-regulated only, (2) down-regulated only, (3) up-down regulated, (4) down-up regulated, (5) up-down-up regulated, (6) down-up-down regulated, (7) up-down-up-down regulated, and (8) down-up-down-up regulated.

Functional classification of identified proteins

Functional annotation of the 196 proteins that were significantly altered after infection of PK-15 cells with PCV2 was initially performed using Protein Center software. Three main annotation types were obtained from the gene ontology consortium Web site: Biological Processes, Subcellular Location, and Molecular Function. Enrichment analysis of biological processes showed that PCV2 infection primarily affects the generation of precursor metabolites and energy (Fig. 4a) and revealed nucleotide-binding, cytoskeletal protein binding, and hydrolyase activity as the commonly affected metabolic functions in PCV2-infected PK-15 cells (Fig. 4c). Furthermore, cellular component-based enrichment analysis showed that proteins with significant alterations are differentially distributed in cells (Fig. 4b).

Protein–protein interactions

Interactions between the virus and host cell are complex and mutual when a virus invades the host. Next, we aimed to determine how PCV2 interacts with PK-15 cell proteins and the effects of these interactions on cell function. The STRING database was searched for interactions with significantly altered proteins in response to PCV2 infection (Fig. 5). The analysis revealed several proteins with interesting interactions, including Hspa12a-Hyou1-Hspa5-Hspd1-Rsp16-Rsp18-Rpl5 and Canx-Calx-Ppib-Ppl10-Prdx2-Anxa2-Anxa1-Vim. These seed proteins play important functions in signal transduction and cell adhesion. For example, Anxa2 is a RNA-binding protein implicated in several cellular transport processes, including internalization and transport of cholesteryl esters, biogenesis of multivesicular bodies, recycling of plasma membrane receptors, and Ca\(^{2+}\)-induced exocytosis of specific secretory granules [24].
Hierarchical cluster analysis for proteins revealed significant alterations in expression levels at different time-courses post-infection. Protein expression is shown using a pseudocolor scale (from $-3$ to $3$), with red indicating high expression and green signifying low expression (Color figure online)
Table 1: Statistically significant differentially expressed proteins identified by iTRAQ analysis of PK-15 cells infected with PCV2

| Accession     | Protein name                                           | Ratio 12 hpi | Ratio 24 hpi | Ratio 36 hpi | Ratio 48 hpi | p value (95%) | % Cov (95%) | Peptides (95%) | Function                                                                 |
|---------------|--------------------------------------------------------|--------------|--------------|--------------|--------------|---------------|--------------|----------------|--------------------------------------------------------------------------|
| gi|34447150 | Heat shock 70 kDa protein 8                            | 0.98         | 1.03         | 1.56         | 2.96         | 4.58E-8       | 45.05        | 43             | Repressor of transcriptional activation                                 |
| gi|346986428 | Heat shock 90kD protein 1                              | 0.88         | 1.11         | 1.18         | 2.13         | 3.59E-7       | 32.73        | 31             | Stress response                                                          |
| gi|30579657 | 78-kDa glucose-regulated protein                        | 1.56         | 1.46         | 1.34         | 1.08         | 1.79E-5       | 37.03        | 31             | Facilitate the assembly of multimeric protein                            |
| gi|35009193 | Prolyl 4-hydroxylase beta polypeptide                   | 1.17         | 1.11         | 1.07         | 1.64         | 6.74E-6       | 37.40        | 28             | Cell redox homeostasis                                                   |
| gi|33282386 | Elongation factor 2                                     | 1.01         | 0.98         | 1.22         | 2.51         | 4.58E-8       | 20.51        | 16             | Translational elongation                                                 |
| gi|33529090 | Annexin A5                                             | 0.99         | 1.18         | 1.56         | 1.14         | 4.58E-8       | 42.37        | 20             | Calcium ion binding                                                      |
| gi|359811347| 60-kDa heat shock protein                              | 1.08         | 1.36         | 1.53         | 1.66         | 5.28E-7       | 23.73        | 19             | Chaperone-mediated protein complex assembly                              |
| gi|350579657| 78-kDa glucose-regulated protein                        | 1.56         | 1.46         | 1.34         | 1.08         | 1.79E-5       | 37.03        | 31             | Facilitate the assembly of multimeric protein                            |
| gi|343478174| T-complex protein 1 subunit alpha                       | 0.81         | 1.32         | 1.38         | 1.24         | 4.68E-7       | 17.51        | 8              | ATP binding                                                              |
| gi|349501107| Ribosomal protein large P subunit                      | 1.36         | 1.10         | 1.74         | 1.42         | 4.34E-7       | 21.70        | 7              | Structural constituent of ribosome                                       |
| gi|35057800 | Ezrin                                                  | 0.86         | 0.81         | 1.32         | 1.87         | 3.51E-8       | 21.21        | 14             | Cytoskeletal anchoring at plasma membrane                                |
| gi|3473575  | Lactate dehydrogenase-B                                | 0.83         | 1.33         | 1.66         | 1.36         | 1.48E-7       | 26.05        | 10             | Cellular carbohydrate metabolic process                                  |
| gi|223019599| Eukaryotic translation elongation factor 1              | 1.00         | 1.16         | 1.42         | 1.72         | 2.11E-7       | 21.00        | 17             | Translation elongation factor activity                                   |
| gi|35017848 | Beta 5-tubulin                                          | 0.79         | 1.08         | 1.72         | 1.58         | 5.91E-8       | 24.32        | 16             | Structural constituent of cytoskeleton                                   |
| gi|358009193| Prolyl 4-hydroxylase beta polypeptide                   | 1.17         | 1.11         | 1.07         | 1.64         | 6.74E-6       | 37.40        | 28             | Cell redox homeostasis                                                   |
| gi|350583022| 40S Ribosomal protein S17                               | 1.00         | 0.87         | 3.25         | 0.99         | 1.19E-9       | 17.04        | 6              | rRNA processing                                                          |
| gi|35058568 | Serine/arginine-rich splicing factor 3                   | 2.29         | 0.78         | 1.31         | 1.07         | 1.13E-8       | 17.68        | 3              | mRNA splicing, via spliceosome                                           |
| gi|35704786 | Peroxiredoxin-6                                        | 0.77         | 1.11         | 1.39         | 1.21         | 4.51E-7       | 41.07        | 18             | Redox regulation of the cell                                             |
| gi|35674889 | Heat shock 70 kDa protein 1B                            | 1.37         | 1.17         | 0.79         | 1.34         | 4.11E-7       | 32.76        | 23             | Stress response                                                          |
| gi|38791290 | Calreticulin                                           | 0.81         | 1.32         | 1.38         | 1.24         | 4.68E-7       | 17.51        | 8              | Calcium ion binding                                                      |
| gi|31125254 | T-complex protein 1 subunit delta                       | 0.94         | 1.26         | 1.33         | 1.58         | 4.30E-7       | 12.80        | 7              | ATP binding                                                              |
| gi|350583022| 14-3-3 protein zeta/delta                              | 0.79         | 1.32         | 1.53         | 2.21         | 1.70E-8       | 38.37        | 13             | Cellular membrane organization                                           |
| gi|358009193| Prolyl 4-hydroxylase beta polypeptide                   | 1.17         | 1.11         | 1.07         | 1.64         | 6.74E-6       | 37.40        | 28             | Cell redox homeostasis                                                   |
| gi|35057968 | Heat shock 70 kDa protein 1B                            | 1.37         | 1.17         | 0.79         | 1.34         | 4.11E-7       | 32.76        | 23             | Stress response                                                          |
| gi|350583022| 14-3-3 protein zeta/delta                              | 0.79         | 1.32         | 1.53         | 2.21         | 1.70E-8       | 38.37        | 13             | Cellular membrane organization                                           |
| gi|350583022| 14-3-3 protein zeta/delta                              | 0.79         | 1.32         | 1.53         | 2.21         | 1.70E-8       | 38.37        | 13             | Cellular membrane organization                                           |
| gi|350583022| 14-3-3 protein zeta/delta                              | 0.79         | 1.32         | 1.53         | 2.21         | 1.70E-8       | 38.37        | 13             | Cellular membrane organization                                           |
| Accession       | Protein name                                      | Ratio          | P value       | % Cov (95%) | Peptides (95 %) | Function                                      |
|----------------|--------------------------------------------------|----------------|---------------|-------------|------------------|-----------------------------------------------|
| gi|335280113 | 60S ribosomal protein L4 | 1.38 | 0.79 | 1.12 | 4.29 | 3.10E–10 | 5.39 | 3 | Translational elongation |
| gi|39618965 | U2 small nuclear RNA auxiliary factor 2 | 0.97 | 1.26 | 0.88 | 1.53 | 2.74E–7 | 3.40 | 2 | Nucleotide binding |
| gi|46644746 | Calmodulin 1 | 1.29 | 1.24 | 4.33 | 1.42 | 4.02E–10 | 34.90 | 11 | Calcium ion binding |
| gi|1273095 | ATP binding cassette sub-family B member 6 | 0.86 | 0.95 | 1.49 | 1.37 | 2.27E–7 | 2.09 | 2 | Cadmium ion transmembrane transport |
| gi|4037946 | Tropomyosin alpha-4 chain | 1.50 | 1.15 | 1.10 | 0.94 | 6.70E–7 | 18.55 | 6 | Muscle filament sliding |
| gi|50583632 | Calponin-3 | 1.37 | 1.22 | 0.90 | 1.56 | 3.44E–7 | 16.72 | 4 | Actomyosin structure organization |
| gi|1940191 | Rho GDP-dissociation inhibitor 1 | 0.78 | 1.06 | 1.61 | 1.71 | 5.35E–8 | 29.90 | 7 | Rho protein signal transduction |
| gi|15502828 | 40S ribosomal protein S3 | 0.83 | 1.10 | 1.46 | 1.61 | 1.34E–7 | 20.16 | 5 | DNA-(apurinic or apyrimidinic site) lyase activity |
| gi|50584132 | Proliferation-associated protein 2G4 | 0.87 | 0.85 | 2.19 | 1.79 | 1.01E–8 | 19.57 | 4 | ERBB3-regulated signal transduction pathway |
| gi|46986388 | Profilin-1 | 0.88 | 1.03 | 0.90 | 1.82 | 5.23E–8 | 52.14 | 9 | Actin cytoskeleton organization |
| gi|47523692 | Thioredoxin | 1.45 | 0.97 | 1.57 | 0.95 | 1.95E–7 | 29.52 | 8 | Possesses a dithiol-reducing activity |
| gi|19403691 | Thiosulfate sulfurtransferase | 2.25 | 0.92 | 1.29 | 1.15 | 1.77E–8 | 24.09 | 3 | Sulfur amino acid catabolic process |
| gi|35059387 | Basic transcription factor 3 | 1.46 | 1.17 | 1.28 | 4.09 | 5.44E–10 | 24.07 | 5 | Transcription regulatory region DNA binding |
| gi|34765897 | ATP synthase, H + transporting, mitochondrial Fo complex | 0.79 | 1.15 | 1.54 | 0.83 | 1.42E–7 | 33.54 | 4 | Mitochondrial ATP synthesis coupled proton transport |
| gi|34627212 | Ribosomal protein L3 | 0.89 | 1.14 | 1.13 | 1.87 | 6.42E–8 | 8.69 | 4 | Structural constituent of ribosome |
| gi|78056236 | RNA-binding protein 4B | 0.88 | 0.88 | 0.82 | 1.47 | 2.36E–7 | 7.52 | 3 | Nucleotide binding |
| gi|4039371 | 40S ribosomal protein S18 | 0.89 | 1.26 | 1.69 | 1.87 | 5.35E–8 | 21.05 | 3 | rRNA binding |
| gi|80747646 | nm23-H2, nucleoside diphosphate kinase B | 0.99 | 1.98 | 1.49 | 1.32 | 7.18E–8 | 24.34 | 3 | GTP biosynthetic process |
| gi|21254962 | 40S ribosomal protein S3a | 0.88 | 1.75 | 1.25 | 0.86 | 6.87E–8 | 7.95 | 2 | Ribonucleoprotein complex |
| gi|21398306 | 60S ribosomal protein L26 | 1.24 | 1.03 | 0.86 | 1.96 | 3.79E–8 | 17.24 | 3 | Ribosomal large subunit biogenesis |
| gi|9190495 | Protein S100-A6 | 2.15 | 0.94 | 1.20 | 1.03 | 2.16E–8 | 16.67 | 2 | Signal transduction |
| gi|4037165 | Guanine nucleotide-binding protein subunit beta-2 | 0.88 | 1.45 | 1.15 | 1.10 | 6.42E–7 | 7.89 | 2 | G-protein coupled receptor signaling pathway |
| gi|3353067 | Protein S100-A2 | 1.27 | 1.17 | 1.91 | 1.10 | 1.09E–7 | 16.49 | 2 | Endothelial cell migration |
| gi|34958507 | Small acidic protein | 1.13 | 0.79 | 1.72 | 1.56 | 6.59E–8 | 21.55 | 7 | Regulation of gene expression, epigenetic |
| gi|50595172 | UPF0562 protein C7orf55 homolog | 1.94 | 1.03 | 1.91 | 0.83 | 1.83E–8 | 8.08 | 4 | Belongs to the UPF0562 family |
| gi|16679606 | 60S ribosomal protein L5 | 1.06 | 1.00 | 1.54 | 1.74 | 1.20E–7 | 6.40 | 2 | Ribosomal large subunit biogenesis |
| gi|35058030 | Far upstream-element-binding protein 2 | 1.07 | 0.85 | 1.61 | 0.91 | 1.45E–7 | 5.76 | 4 | mRNA processing |
| gi|1709973 | 60S ribosomal protein L10a | 1.63 | 1.03 | 1.43 | 1.43 | 5.18E–7 | 13.33 | 3 | RNA binding |
| gi|75073672 | Calcium/calmodulin-dependent protein kinase type II | 1.06 | 1.10 | 1.77 | 2.65 | 1.52E–9 | 5.81 | 2 | Calcium ion transport |
| gi|35059633 | Copper transport protein ATOX1 | 1.33 | 0.78 | 1.96 | 1.31 | 3.83E–8 | 31.82 | 2 | Response to oxidative stress |
| Accession | Protein name | Ratio  | P value  | % Cov | Peptides (95%) | Function                                      |
|-----------|--------------|--------|----------|-------|----------------|-----------------------------------------------|
| gi|178056781 | Histone H2A.Z | 0.85 | 1.34E−7 | 31.25 | 6 | Nucleosome assembly |
| gi|58517860 | Thymosin beta-10 | 1.43 | 1.80E−6 | 50.00 | 5 | Actin cytoskeleton organization |

**Cluster 2: down-regulation (49)**

| Accession | Protein name | Ratio  | P value  | % Cov | Peptides (95%) | Function                                      |
|-----------|--------------|--------|----------|-------|----------------|-----------------------------------------------|
| gi|311273021 | Fibronecin isoform 2 | 0.70 | 1.24E−6 | 13.20 | 30 | Matrix organization of cartilage |
| gi|47523618 | Citrate synthase | 0.89 | 7.18E−7 | 7.76 | 3 | ATP catabolic process |
| gi|311267276 | Keratin type I cytoskeletal 19 | 0.58 | 1.16E−6 | 56.19 | 27 | Organization of myofibers |
| gi|227430407 | Keratin, type II cytoskeletal 8 | 0.60 | 1.44E−7 | 33.54 | 21 | Cell morphogenesis involved in differentiation |
| gi|347300243 | Glutamate dehydrogenase 1 | 0.43 | 3.42E−7 | 24.01 | 14 | Cellular amino acid metabolic process |
| gi|57279735 | Succinate dehydrogenase [ubiquinone] iron–sulfur subunit | 0.59 | 1.62E−7 | 6.07 | 2 | Electron transport |
| gi|55983054 | Proteasome 26S subunit non-ATPase 4 | 1.21 | 1.02E−7 | 11.14 | 2 | mRNA metabolic process |
| gi|350594261 | NAD(P) transhydrogenase | 0.27 | 2.19E−7 | 2.19 | 2 | Reactive oxygen species metabolic process |
| gi|343887420 | Transcription elongation factor A protein 1 | 1.14 | 4.11E−7 | 12.95 | 3 | Rho protein signal transduction |
| gi|5739517 | Macrophage migration inhibitory factor | 1.13 | 4.51E−7 | 16.22 | 4 | Pro-inflammatory cytokine |
| gi|219522018 | Na[+]/H[+] exchange regulatory cofactor NHE-RF1 | 0.99 | 2.46E−6 | 6.85 | 2 | Wnt receptor signaling pathway |
| gi|346986432 | ras homolog gene family, member A | 0.69 | 1.98E−6 | 12.95 | 3 | Rho protein signal transduction |
| gi|350594505 | Annexin A6 | 0.44 | 4.30E−7 | 3.79 | 3 | Regulate the release of Ca²⁺ from intracellular stores |
| gi|57527987 | Moesin | 0.93 | 3.63E−6 | 20.80 | 13 | Structural constituent of cytoskeleton |
| gi|335284397 | Major vault protein isoform 1 | 0.64 | 1.12E−6 | 18.56 | 15 | Protein transport |
| gi|335305558 | Heterogeneous nuclear ribonucleoproteins A2/B1 isoform 1 | 1.09 | 4.97E−7 | 43.91 | 16 | Pre-mRNA intronic binding |
| gi|393714792 | Sodium/potassium-transporting ATPase subunit alpha-1 | 0.65 | 2.62E−7 | 17.45 | 16 | Sodium/potassium transport |
| gi|311261216 | c-1-tetrahydrofolate synthase | 0.54 | 5.65E−5 | 10.37 | 8 | Formate-tetrahydrofolate ligase activity |
| gi|335306989 | ATP-dependent RNA helicase A | 0.45 | 1.26E−6 | 5.99 | 6 | Putative ATP-dependent RNA helicase |
| gi|335299026 | Microtubule-associated protein 4 | 1.11 | 2.43E−7 | 6.57 | 5 | Promotes microtubule assembly |
| gi|350582932 | Annexin A13 | 0.60 | 4.82E−7 | 19.63 | 6 | Calcium ion binding |
| gi|50403675 | Vinculin | 0.65 | 2.99E−7 | 6.43 | 6 | Cell-matrix adhesion and cell-cell adhesion |
| gi|350535040 | Eukaryotic translation initiation factor 4 gamma 1 | 0.82 | 9.76E−7 | 4.13 | 6 | Regulation of translational initiation |
| gi|329663948 | ras GTPase-activating protein-binding protein 1 | 0.96 | 4.37E−7 | 15.27 | 7 | ATP-dependent DNA helicase activity |
| gi|178056550 | D-3-phosphoglycerate dehydrogenase | 0.65 | 2.23E−6 | 9.76 | 4 | Amino acid biosynthesis |
| Accession | Protein name | Ratio   | P value | % Cov | Peptides (95%) | Function                                      |
|-----------|--------------|---------|---------|-------|----------------|-----------------------------------------------|
| gi|311254317   | Cingulin | 0.39    | 0.68   | 0.57 | 0.68          | 5.91E–6, 3.18, 4, Cell junction               |
| gi|61216107    | Adenosylhomocysteinase | 0.57 | 1.20   | 0.77 | 0.94          | 4.35E–7, 11.34, 5, S-adenosylhomocysteine catalytic process |
| gi|347300176   | Peroxiredoxin-2 | 0.59 | 0.65   | 0.71 | 0.95          | 3.34E–6, 18.69, 5, Involved in redox regulation of the cell |
| gi|311264042   | Hypoxia up-regulated protein 1 | 1.08 | 1.14   | 0.53 | 1.07          | 3.15E–7, 4.81, 5, A molecular chaperone and participate in protein folding |
| gi|194441525   | Dihydronicotinamide-related protein 2 | 0.26 | 0.94   | 0.56 | 0.79          | 2.76E–7, 7.24, 3, Axon guidance                |
| gi|172072661   | tRNA-splicing ligase RtcB homolog | 1.09 | 1.10   | 0.66 | 0.50          | 1.00E–6, 7.33, 3, tRNA splicing, via endonucleolytic cleavage and ligation |
| gi|347300323   | Thioredoxin-dependent peroxide reductase | 1.06 | 0.47   | 1.25 | 1.14          | 1.40E–7, 17.62, 4, Involved in redox regulation of the cell |
| gi|194037005   | Ribonuclease UK114 | 1.09 | 0.26   | 0.33 | 0.73          | 9.86E–8, 38.69, 4, Nucleic acid phosphodiester bond hydrolysis |
| gi|350594033   | UDP-glucuronosyltransferase | 0.54 | 0.83   | 0.75 | 1.13          | 5.82E–7, 6.60, 3, Glucuronosyltransferase activity |
| gi|75039721    | Unconventional myosin-VI | 0.64 | 0.58   | 0.57 | 0.74          | 5.54E–5, 4.23, 4, Actin-based motor molecules with ATPase activity |
| gi|350595577   | Spermine synthase | 0.89 | 0.64   | 0.93 | 1.16          | 1.01E–6, 9.90, 3, Spermine biosynthetic process |
| gi|7939586     | Dihydrolipoamide succinyltransferase | 0.72 | 1.05   | 0.65 | 0.91          | 1.89E–6, 4.61, 2, Cellular nitrogen compound metabolic process |
| gi|335284690   | Periplakin | 0.25 | 1.24   | 1.16 | 0.95          | 5.06E–8, 1.71, 3, Structural constituent of cytoskeleton |
| gi|350585766   | Chloride intracellular channel protein 4 | 0.63 | 0.70   | 0.86 | 1.11          | 1.01E–6, 25.19, 5, Branching morphogenesis of an epithelial tube |
| gi|350587647   | Hepatoma-derived growth factor | 0.53 | 1.10   | 0.75 | 0.92          | 5.95E–7, 20.58, 3, Acts as a transcriptional repressor |
| gi|273463176   | Cell division cycle 2 variant 1 | 0.79 | 0.66   | 0.62 | 0.27          | 8.53E–7, 11.45, 3, Regulation of transcription, DNA-dependent |
| gi|311272155   | Activated RNA polymerase II transcriptions coactivator p15 | 0.98 | 0.54   | 1.10 | 0.65          | 4.19E–7, 25.98, 4, Transcription, DNA-dependent |
| gi|94441332    | Putative aldo–keto reductase family 1 member C4 | 0.45 | 0.90   | 0.97 | 1.18          | 2.19E–7, 8.70, 2, Oxido reductase activity |
| gi|311250313   | Histidyl-tRNA synthetase | 0.59 | 0.75   | 1.07 | 0.60          | 8.26E–7, 3.93, 2, tRNA aminocacylation for protein translation |
| gi|540049123   | 60S ribosomal protein L22 | 0.88 | 1.15   | 0.94 | 0.43          | 2.47E–7, 18.75, 2, Translation                |
| gi|311250313   | poly(rC)-binding protein 2 | 1.03 | 0.77   | 0.94 | 0.67          | 2.97E–6, 8.78, 4, Immunity                   |
| gi|335300686   | Carboxyl reductase [NADPH] 1 | 0.64 | 0.64   | 1.09 | 0.99          | 6.87E–7, 11.03, 3, Carboxyl reductase (NADPH) activity |
| gi|335459573   | Clathrin light chain (CLTA) protein | 1.25 | 0.55   | 0.87 | 0.91          | 3.09E–7, 33.54, 7, Cellular membrane organization |
| gi|340007404   | Alpha-actin-1 | 0.97 | 0.99   | 0.91 | 0.54          | 1.04E–6, 12.29, 10, Actin cross-link formation |

**Cluster 3: up-down regulation (22)**

| Accession | Protein name | Ratio   | P value | % Cov | Peptides (95%) | Function                                      |
|-----------|--------------|---------|---------|-------|----------------|-----------------------------------------------|
| gi|51592135    | Cofilin-1 | 1.58    | 0.55   | 1.00 | 1.22          | 3.39E–8, 43.98, 11, Regulates actin cytoskeleton dynamics |
| gi|90200404    | Triosephosphate isomerase 1 | 1.26 | 0.56   | 0.86 | 0.84          | 3.02E–7, 17.34, 5, Triosephosphate isomerase activity |
| gi|350584416   | Parathymosin | 1.29 | 0.91   | 0.65 | 0.95          | 4.36E–7, 22.55, 3, Mediate immune function     |
| gi|335309827   | Nuclear autoantigenic sperm protein | 2.07 | 2.27   | 0.96 | 0.69          | 5.38E–9, 7.71, 4, Required for DNA replication |
| gi|335284315   | RNA-binding protein FUS isoform 2 | 1.27 | 0.95   | 0.47 | 0.68          | 1.45E–7, 4.44, 2, mRNA splicing, via spliceosome |
| gi|350579350   | Stomatin-like protein 2 | 0.87 | 1.43   | 1.09 | 0.43          | 6.82E–8, 7.93, 3, T cell receptor signaling pathway |
| Accession      | Protein name                              | Ratio    | P value  | % Cov (95%) | Peptides (95 %) | Function                                                                 |
|---------------|-------------------------------------------|----------|----------|-------------|-----------------|--------------------------------------------------------------------------|
| gi|350594172   | Brain acid soluble protein 1              | 1.45     | 0.58     | 0.24         | 0.90            | 2.97E–8 Glomerular visceral epithelial cell differentiation               |
| gi|335295652   | Laminin subunit beta-1                    | 1.64     | 0.89     | 0.67         | 0.85            | 1.75E–5 Cell migration                                                  |
| gi|346644699   | Protein SET                               | 1.36     | 0.93     | 2.03         | 0.46            | 1.05E–8 Involved in apoptosis, transcription, nucleosome assembly and histone chaperoning |
| gi|345199274   | Glutaredoxin 3                            | 1.33     | 0.82     | 2.38         | 0.65            | 5.65E–9 Protein disulfide oxidoreductase activity                        |
| gi|345059120   | 26S proteasome non-ATPase regulatory subunit 6 | 1.25  | 2.33     | 1.69         | 0.63            | 7.88E–9 Transcription coactivator activity                              |
| gi|162951821   | Heterogeneous nuclear ribonucleoprotein A/B | 1.24  | 1.33     | 0.67         | 1.24            | 2.47E–7 Binds single-stranded RNA                                       |
| gi|346716324   | Myosin regulatory light chain 2 protein isoform 2 | 1.54  | 0.69     | 1.03         | 0.86            | 1.14E–7 Cardiac myofibril assembly                                       |
| gi|311249564   | Heterogeneous nuclear ribonucleoprotein H | 1.85     | 1.20     | 1.12         | 0.10            | 7.61E–9 Regulation of RNA splicing                                       |
| gi|311247963   | Apoptosis inhibitor 5 isoform 1            | 1.04     | 1.63     | 0.18         | 0.53            | 1.31E–8 Apoptosis                                                       |
| gi|345059135   | Hypothetical protein LOC100522278          | 1.46     | 0.79     | 0.88         | 0.67            | 1.31E–7 None                                                            |
| gi|38921635    | FKBP1A                                    | 1.47     | 0.77     | 0.62         | 1.09            | 1.03E–7 Beta-amyloid formation                                           |
| gi|75069665    | ADP-ribosylation factor-like protein 3     | 0.75     | 1.74     | 1.28         | 0.69            | 3.50E–8 Cilium morphogenesis                                             |
| gi|48675927    | Tropomyosin alpha-3 chain                 | 2.19     | 0.70     | 0.90         | 1.04            | 1.03E–8 SH3 domain-binding glutamic acid-rich-like protein               |
| gi|350595802   | SH3 domain-binding glutamic acid-rich-like protein | 1.04  | 1.05     | 1.38         | 0.48            | 1.07E–7 SH3/SH2 adaptor activity                                         |
| gi|194034833   | Microfibrillar-associated protein 1        | 0.91     | 1.41     | 0.53         | 0.79            | 1.12E–7 Extracellular matrix organization                               |
| gi|311250943   | 28 kDa heat- and acid-stable phosphoprotein | 1.18  | 1.29     | 1.46         | 0.62            | 1.18E–7 Enhances PDGFA-stimulated cell growth in fibroblasts             |
| Clustering 4: down-up regulation (43) | | | | | | |
| gi|408360214   | Vimentin                                   | 0.97     | 0.57     | 0.55         | 1.58            | 3.85E–8 Class-III intermediate filaments                                 |
| gi|28948618    | Annexin A1                                 | 0.34     | 1.22     | 1.64         | 2.91            | 1.59E–9 Calcium/phospholipid-binding protein                             |
| gi|35059135    | Histone H2B type 1                         | 0.69     | 1.21     | 1.41         | 1.00            | 1.29E–7 Nucleosome assembly                                             |
| gi|350578257   | Heterogeneous nuclear ribonucleoprotein Q isoform 1 | 0.75  | 1.25     | 0.69         | 3.70            | 5.06E–10 mRNA processing                                                |
| gi|35059284210 | HSP 27                                     | 0.74     | 0.99     | 1.49         | 1.26            | 1.94E–7 Stress resistance and actin organization                         |
| gi|3505908027  | Elongation factor 1-gamma                  | 0.53     | 0.85     | 1.14         | 1.28            | 1.70E–7 Translation elongation factor activity                           |
| gi|194043605   | GTP-binding nuclear protein Ran            | 0.59     | 1.31     | 0.99         | 2.19            | 9.64E–9 GTP-binding protein involved in nucleocytoplasmic transport    |
| gi|9857227     | Ribophorin 1                               | 0.39     | 0.74     | 1.49         | 0.88            | 4.69E–8 Glycogen transferase activity                                   |
| gi|311245228   | Serpin B5                                  | 0.44     | 1.06     | 1.60         | 0.95            | 4.06E–8 Tumor suppressor                                                |
| gi|350591497   | ras-related protein Rab-7a                | 1.13     | 0.65     | 1.41         | 1.13            | 2.09E–7 Key regulator in endolysosomal trafficking                       |
| Accession  | Protein name                                      | Ratio          | P value | % Cov (95%) | Peptides (95 %) | Function                                                                 |
|-----------|--------------------------------------------------|----------------|---------|-------------|-----------------|--------------------------------------------------------------------------|
| gi|255683404 | Isocitrate dehydrogenase                          | 0.48           | 1.33    | 1.24        | 0.99            | 9.83E-8                                                                 |
| gi|343807407 | Thio|purine S-|methyltransfe|rse                   | 0.68           | 0.56    | 1.72        | 1.60            | 1.56E-8                                                                 |
| gi|35281298  | Tubulin beta-2C chain                             | 0.87           | 0.65    | 1.43        | 0.79            | 1.51E-7                                                                 |
| gi|87455552  | Voltage-dependent anion channel 1                | 0.62           | 1.69    | 1.21        | 1.08            | 5.55E-8                                                                 |
| gi|304654628 | Protein disulfide-isomerase A3                    | 1.11           | 0.54    | 1.53        | 1.11            | 7.62E-8                                                                 |
| gi|87455554  | Voltage-dependent anion channel 2                | 0.72           | 0.65    | 1.27        | 1.42            | 9.28E-8                                                                 |
| gi|94043450  | Nidogen-2                                        | 0.30           | 1.69    | 1.18        | 1.03            | 1.91E-8                                                                 |
| gi|75056558  | Ras-related protein Rab-11A                      | 0.41           | 0.69    | 2.13        | 1.69            | 4.84E-9                                                                 |
| gi|346986294 | Uncharacterized protein LOC100155717 isoform 2     | 0.46           | 0.64    | 1.45        | 1.87            | 1.04E-8                                                                 |
| gi|311245734 | Hypothetical protein LOC100038023                | 1.00           | 0.69    | 1.18        | 2.17            | 6.97E-9                                                                 |
| gi|47522630  | Aspartate aminotransferase                        | 0.61           | 1.21    | 1.06        | 1.85            | 3.01E-8                                                                 |
| gi|362271216 | Ribosomal protein L15                            | 0.95           | 0.74    | 1.39        | 2.09            | 2.92E-8                                                                 |
| gi|311248936 | Phenylalanine-1RNA synthetase alpha chain         | 0.50           | 0.35    | 0.91        | 1.38            | 2.14E-8                                                                 |
| gi|345441771 | Aldolase C, fructose-bisphosphate                 | 0.61           | 1.05    | 1.43        | 1.06            | 1.64E-7                                                                 |
| gi|213958699 | Glutamine:fructose-6-phosphate amidotransferase 1 variant 2 | 0.34           | 4.13    | 1.07        | 5.11            | 7.15E-11                                                                 |
| gi|281500757 | Porcine Aldehyde Reductase In Ternary Complex With Inhibitor | 0.30           | 0.74    | 1.11        | 1.58            | 2.37E-8                                                                 |
| gi|335284299 | T-complex protein 1 subunit zeta                  | 1.05           | 0.98    | 0.71        | 1.56            | 1.31E-7                                                                 |
| gi|311247012 | Epidermal growth factor receptor kinase substrate 8 | 1.14           | 0.25    | 1.28        | 0.89            | 4.81E-8                                                                 |
| gi|60394813  | 40S ribosomal protein S16                         | 1.18           | 1.15    | 0.65        | 4.61            | 1.87E-10                                                                 |
| gi|362272222 | Ribosomal protein L13a isoform 1                  | 0.82           | 0.52    | 1.96        | 0.92            | 1.34E-8                                                                 |
| gi|36421378  | Serpin H1                                        | 0.95           | 0.59    | 0.99        | 2.29            | 6.74E-9                                                                 |
| gi|324349319 | Calnexin                                         | 0.14           | 0.90    | 2.33        | 1.50            | 2.81E-9                                                                 |
| gi|36644805  | Pinin                                            | 0.63           | 1.57    | 1.33        | 1.14            | 8.19E-8                                                                 |
| gi|85681889  | 60S ribosomal protein L10                         | 0.86           | 1.20    | 0.60        | 1.89            | 2.13E-8                                                                 |
| gi|311252000 | Fumarlylactoacetate hydrolase domain-containing protein 2 | 1.16           | 0.99    | 0.52        | 2.83            | 2.02E-9                                                                 |
| gi|54020966  | Annexin A2                                       | 0.74           | 1.11    | 1.11        | 2.61            | 4.38E-9                                                                 |
| gi|350585373 | Myosin-14                                        | 0.72           | 0.77    | 2.25        | 0.76            | 6.57E-9                                                                 |
| gi|51870491  | CDC37 cell division cycle 37 protein              | 0.74           | 1.12    | 1.37        | 1.06            | 4.61E-7                                                                 |
| gi|85792232 | Eukaryotic translation initiation factor 4A isoform 1 | 0.72           | 1.79    | 1.26        | 0.89            | 4.13E-8                                                                 |
| Accession | Protein name | Ratio 12 hpi | Ratio 24 hpi | Ratio 36 hpi | Ratio 48 hpi | P value | % Cov (95%) | Peptides (95 %) | Function |
|-----------|--------------|--------------|--------------|--------------|--------------|---------|-------------|----------------|----------|
| gi|4033507 | Annexin A4 | 0.71 | 1.45 | 1.54 | 1.09 | 1.01E–7 | 33.54 | 11 | Calcium/phospholipid-binding protein |
| gi|343403779 | Ribosomal protein L13 | 1.24 | 0.70 | 1.18 | 1.50 | 1.68E–7 | 7.11 | 2 | Translational elongation |
| gi|45268967 | 40S ribosomal protein S28 | 1.17 | 0.74 | 1.51 | 0.77 | 1.16E–7 | 26.25 | 4 | Translational elongation |
| gi|343432604 | Ubiquitin-conjugating enzyme E2 variant 2 | 0.71 | 1.98 | 1.29 | 1.38 | 2.85E–8 | 21.38 | 2 | Error-free post-replication DNA repair |

**Cluster 5: up-down-up regulation (6)**

| Accession | Protein name | Ratio 12 hpi | Ratio 24 hpi | Ratio 36 hpi | Ratio 48 hpi | P value | % Cov (95%) | Peptides (95 %) | Function |
|-----------|--------------|--------------|--------------|--------------|--------------|---------|-------------|----------------|----------|
| gi|350588024 | Heterogeneous nuclear ribonucleoprotein D0 | 1.09 | 1.37 | 0.74 | 1.27 | 3.53E–7 | 16.42 | 4 | RNA catabolic process |
| gi|350586335 | Nuclease-sensitive element-binding protein 1 | 1.27 | 0.77 | 0.95 | 2.17 | 1.39E–8 | 20.95 | 6 | Mediates pre-mRNA alternative splicing regulation |
| gi|350585579 | Alpha-enolase | 1.39 | 0.61 | 2.15 | 1.18 | 1.26E–8 | 18.21 | 13 | Magnesium ion binding |
| gi|85542092 | 60S ribosomal protein L6 | 1.60 | 0.59 | 1.43 | 2.21 | 1.04E–8 | 9.51 | 3 | Translation |
| gi|194037373 | Coatomer subunit beta-1 isoform 1 | 3.10 | 0.59 | 3.56 | 1.11 | 4.58E–10 | 18.64 | 2 | Intracellular protein transport |
| gi|350594189 | Cadherin-10 | 1.60 | 1.38 | 0.63 | 1.46 | 5.83E–8 | 3.16 | 2 | Calcium ion binding |

**Cluster 6: down-up-down regulation (11)**

| Accession | Protein name | Ratio 12 hpi | Ratio 24 hpi | Ratio 36 hpi | Ratio 48 hpi | P value | % Cov (95%) | Peptides (95 %) | Function |
|-----------|--------------|--------------|--------------|--------------|--------------|---------|-------------|----------------|----------|
| gi|342672022 | exportin-2 | 0.74 | 0.64 | 1.32 | 0.47 | 1.12E–7 | 3.60 | 4 | cell proliferation |
| gi|350583346 | ubiquitin associated protein 2-like isoform 3 | 0.57 | 0.94 | 3.31 | 0.56 | 3.86E–10 | 3.04 | 2 | binding of sperm to zona pellucida |
| gi|335309813 | coactosin-like protein | 0.96 | 0.68 | 1.26 | 0.26 | 6.36E–8 | 12.50 | 2 | Binds to F-actin in a calcium-independent manner |
| gi|350578507 | asparaginyl-tRNA synthetase | 0.94 | 0.41 | 1.63 | 0.73 | 2.92E–8 | 6.80 | 4 | tRNA aminoacylation for protein translation |
| gi|350596594 | malate dehydrogenase | 0.56 | 0.57 | 1.28 | 0.53 | 1.18E–7 | 20.76 | 7 | L-malate dehydrogenase activity |
| gi|60389340 | m7GpppX diphosphatase | 0.56 | 1.43 | 0.82 | 0.65 | 8.83E–8 | 10.09 | 2 | cellular response to menadione |
| gi|350592411 | proteasome (prosome, macropain) 26S subunit, ATPase, 2 | 0.51 | 2.73 | 2.91 | 0.61 | 7.12E–10 | 13.63 | 5 | enzyme regulator activity |
| gi|51317314 | Histone H4 | 0.07 | 1.94 | 0.28 | 1.19 | 3.75E–9 | 50.49 | 7 | Core component of nucleosome |
| gi|1940444626 | 14-3-3 protein beta/alpha isoform 1 | 1.04 | 0.69 | 1.54 | 0.65 | 7.19E–8 | 32.52 | 8 | Ras protein signal transduction |
| gi|350578528 | peptidyl-prolyl cis-trans isomerase B | 0.72 | 0.62 | 1.34 | 0.29 | 5.62E–8 | 23.20 | 5 | PPases accelerate the folding of proteins |
| gi|355282599 | interleukin enhancer-binding factor 3 | 0.30 | 0.70 | 2.83 | 0.58 | 1.13E–9 | 11.95 | 6 | Transcription regulation |

**Cluster 7: up-down-up-down regulation (1)**

| Accession | Protein name | Ratio 12 hpi | Ratio 24 hpi | Ratio 36 hpi | Ratio 48 hpi | P value | % Cov (95%) | Peptides (95 %) | Function |
|-----------|--------------|--------------|--------------|--------------|--------------|---------|-------------|----------------|----------|
| gi|350290365 | 45 kDa calcium-binding protein | 1.39 | 0.59 | 1.45 | 0.48 | 2.99E–8 | 4.48 | 2 | calcium ion binding |

**Cluster 8: down-up-down-up regulation (1)**

| Accession | Protein name | Ratio 12 hpi | Ratio 24 hpi | Ratio 36 hpi | Ratio 48 hpi | P value | % Cov (95%) | Peptides (95 %) | Function |
|-----------|--------------|--------------|--------------|--------------|--------------|---------|-------------|----------------|----------|
| gi|350594669 | ribosome-binding protein 1 | 0.69 | 1.64 | 0.63 | 1.92 | 1.1E–8 | 4.25 | 5 | rRNA processing |

The 196 unique proteins identified with 95% confidence (corresponding to a protein score cutoff >1.3), were clustered based on similar trends of differential 489 expression over times. % Cov (95) means percent coverage (95%). The proteins were considered to show a significant upward or downward trend if their expression ratios relative to the mock control group at the same time post infection were >1.25 or <0.75, respectively. One-way analysis of variance (ANOVA) and LSD analysis (SPSS 18.0) were used in this statistical test. Differences between groups through the time course were considered statistically significant for P values < 0.05.
Validation of changes in protein levels via Western blot analysis.

To validate the differentially expressed proteins identified using the iTRAQ labeled LC–MS/MS system, vimentin, Annexin I, Hsp90, and Rab-11A were selected for Western blot analysis. Equal amounts of cell lysate protein from PCV2-infected PK-15 and virus-free cells were tested with antibodies against vimentin, AnnexinI, Hsp90, and Rab-11A, respectively. As shown in Fig. 6, the Hsp90 was up-regulated at 96 hpi. In addition, the expression of vimentin, Annexin I, Hsp90, and Rab-11A were selected for Western blot analysis.
AnnexinI, and Rab-11A showed down-regulation in the prophase of infection, and then, they up-regulated later. The ratios of the four representative proteins between infected and uninfected cells were consistent with those obtained from iTRAQ-coupled 2D LC–MS/MS analysis. Protein spot levels were determined using ImageJ quantification software.

**Discussion and conclusions**

Upon virus infection, cellular environments are modified to eliminate the invading virus by host antiviral responses or to favor virus replication by viral evasion strategies. The changes of host gene production in virus-infected cells have been largely studied to elucidate pathogenic mechanism associated with such alterations. However, very limited information is currently available for cellular protein productions regulated after exposed to individual viral components. To further elucidate the molecular mechanisms involved in PCV2 infection of host cells, we screened the differentially expressed proteins associated with PK-15 cells infected with the virus using comparative proteomics. Several earlier studies have analyzed the interplay between PCV2 and host cells using proteomics analysis, which includes interactions of PCV2 and PK-15

**Fig. 5** Protein–protein interaction network analyzed via STRING software. An edge was drawn with up to seven different colored lines representing the presence of seven lines of evidence used in predicting associations. A red line indicates the presence of fusion evidence, a green line indicates neighborhood evidence, a blue line indicates co-occurrence evidence, a purple line indicates experimental evidence, a yellow line indicates text mining evidence, a light blue line indicates database evidence, and a black line indicates coexpression evidence (Color figure online)
cells [15, 16], porcine alveolar macrophages (PAMs) [17, 18], and inguinal lymph nodes of piglets inoculated with PCV2 [14]. In order to determine further virus–host interactions and the processes leading to disease onset, a high-throughput quantitative proteomic approach, iTRAQ was utilized to investigate the differential proteomes of a highly permissive PK-15 clone cells in response to PCV2 infection. The results indicated that a total of 196 proteins displayed significantly altered expression at different time points post-infection. Four of these proteins were confirmed to be regulated in PCV2-infected PK-15 cells using immunoblotting as an independent analytical method. It provided critical clues for further analysis of PCV2 pathogenesis.

Proteomics is a novel methodology employed to detect the components of cellular protein interactions as well as host cellular pathophysiological processes that occur during virus infection [25, 26]. iTRAQ, combined with LC and tandem MS analysis, is emerging as a powerful technology in the search for disease-specific targets. This procedure is ideally suited to our study, since it allows the comparison of four time points after infection and four corresponding controls. Compared with mock infection, PCV2 did not induce visible cytopathic effects. Furthermore, the full cell monolayer appeared in PCV2-infected cells. Both IFA and Western blot results disclosed that PCV2 replicates in PK-15 cells. Differentially expressed proteins are involved in cytoskeleton organization, macromolecular biosynthesis, signal transduction, stress response, ubiquitin–proteasome pathway (UPP), and metabolic enzymes (Table 1). Our data aid in the understanding of the pathogenesis of PCV2 infection. In our previous quantitative proteomics study, we identified that Hsp70 was up-regulated in PCV2-infected PAMs [18]. Then, we firstly found that Hsp70 could positively regulate PCV2 replication in a continuous porcine monocytic cell line 3D4/31 [27]. It can be seen that the results of proteomics study should be useful to elucidate the mechanism of replication of PCV2 in the future.

The host cytoskeletal network participates in the transport of viral components in the cell, particularly during the stages of the entry and exit of the virus [28]. Our data strongly indicate an important role for cytoskeletal proteins in PCV2 infection in PK-15 cells. The identified microfilament-associated and microtubule-associated proteins, i.e., annexin A2 and beta 5-tubulin, were up-regulated (Table 1), whereas the microtubule-associated protein 4, alpha-actin, and keratin 8 were down-regulated in the process of infection (Table 1). The existing evidence suggests that actin can regulate gene transcription in virus-induced signaling. The mRNA-binding activity of actin is important for the anchoring, transporting, and topological positioning of mRNAs [29]. With regard to beta-tubulin, there are several reports showing that viruses may require microtubule components for RNA synthesis [30]. Actin was identified as being overexpressed when it interacts with Rep of PCV2 through colocalization and coimmunoprecipitation analyses [15]. Changes in beta-tubulin and vimentin levels have been detected in SARS-CoV22 and infectious bursal disease virus (IBDV) [26, 31]. However, Fan et al. [16.] indicated that beta-tubulin level was down-regulated in infected PK-15 cells and speculated that the vimentin and beta-tubulin networks collapse and disperse in host cells, leading to an unstable cytoskeletal structure and release of viral particles from the infected cells. The roles of these cytoskeletal proteins in PK15 cells after PCV2 infection should be further investigated.

Heat shock proteins (HSPs) are a class of multifunctional proteins that maintain cell stability when cells are exposed to elevated temperatures, pathogens, and/or other environmental stresses [32]. Activation of the heat shock response might be a specific virus function that ensures proper synthesis of viral proteins and virions; thus, stress...
proteins may be important for virus replication [33]. Mammalian cells have developed response networks which detect and control diverse forms of stress. One of these responses, known as heat shock response, is a universal mechanism necessary for cell survival under a variety of unfavorable conditions like virus infections [34]. In the present study, the up-regulation of Hsp90, Hsp70, and Hsp60 was identified in the PCV2-infected PK-15 cells (Table 1). Hsp60 is a mitochondrial chaperonin that is typically responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix, and it was reported that Hsp60 folds 30% of the cytoplasmic proteins under heat stress [35]. One study reported that Hsp60 can directly activate leukocytes, epithelial cells, and fibroblasts to secrete proinflammatory cytokines such as TNF and interleukins, which participate in the process of T cell-mediated immunity [36]. It has been reported that HSPs inhibit the replication of IV and a variety of RNA viruses [37]. Inhibition of the expression of Hsc70 blocks the nuclear export of the IV M1 and NP proteins and thereby inhibits the production of the progeny virus [37].

Previously, we demonstrated that Hsp70 has a positive regulatory effect on PCV2 infection cycle, based on proteomics results on PCV2-infected PAMs [27]. Hsp90 has a very important function in the folding of cell regulatory proteins and the refolding of stress-denatured polypeptides [38–40]. Prior research has also shown that Hsp90 is involved in the assembly and nuclear export process of IV RNPs [41]. A previous report showed that an initial increase in Hsp90 expression at 12 h after infection suggests a cellular response to hMPV-induced ER stress initiated by an increase in unfolded or misfolded proteins [42]. Recently, an association between Hsp90 protein complex and lamina A/C has been observed after oxidative stress [43]. Also, in our PCV2-infected PK-15 cells, we observe an increase in Hsp90 and lamin subunit beta. Here, the increased information should be helpful to study on the molecular mechanisms underlying host–PCV2 interactions.

Several signal transduction-associated proteins were identified to be differentially expressed in the PCV2-infected cells. The 14-3-3 protein family is known to be involved in the regulation of several signal transduction pathways including those regulating the cell cycle, apoptosis, cytoskeletal remodeling, transcription, and stress responses [44]. A prior study reported that the 14-3-3 protein interacts with the HCV core protein to activate kinase Raf-1 [45], and the identified interaction of the 14-3-3 protein with Vpr has a functional significance for cell cycle regulation in HIV-1 infection [46]. In our study, the expression of 14-3-3 protein in PCV2-infected PK-15 cells was down-regulated. This occurrence may be due to the cellular physiology dysfunction of PK-15 cells. Further work is clearly necessary to examine the function of 14-3-3 protein in PCV2-infected tissues.

Ubiquitin–proteasome pathway (UPP), a major intracellular protein degradation pathway, has recently been implicated in viral infections, including avoidance of host immune surveillance, viral maturation, viral progeny release, efficient viral replication, and reactivation of virus from latency [47]. Some viruses have been reported to evolve different strategies to utilize the UPP for beneficial reasons, including the indication that ubiquitin–proteasome system is required for avoidance of host immune surveillance during HIV-1 and is necessary for transcriptional regulation of the DNA virus, herpes simplex virus (HSV) [48, 49]. Mumps virus and simian virus inhibit JAK/STAT signaling pathway through proteasome degradation of the cellular STAT protein to escape the interferon-initiated antiviral responses [50]. In this study, PCV2 infection induced expression of the ubiquitin-conjugating enzyme E2, proteasome 26S subunit, and ubiquitin-associated protein 2. Therefore, their change in abundance levels may indicate to an important pathway affected by PCV2 replication. Whether PCV2 takes the similar or different strategy during infection has not been elucidated and deserves further investigation.

In summary, in this study, an iTRAQ proteomics approach was adopted to probe differentially expressed proteins in PCV2-infected PK-15 cells. Using unambiguous methods, we identified 196 cellular proteins that were significantly altered following PCV2 infection. The abundance of differentially expressed proteins should aid in elucidating molecular mechanisms associated with interactions between PCV2 and target cells. However, the proteomics results were preliminary data, which needed to be further elaborated and analyzed for understanding the roles of these proteins in PCV2 infection.

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