Profiling and functional analysis of differentially expressed circular RNAs identified in foot-and-mouth disease virus infected PK-15 cells

JinKe Yang, Bo Yang, Yong Wang, Ting Zhang, Yu Hao, HuiMei Cui, DengShuai Zhao, XingGuo Yuan, XueHui Chen, ChaoChao Shen, WenQian Yan, HaiXue Zheng*, KeShan Zhang* and Xiangtao Liu

Abstract
Circular RNAs (circRNAs) are a new type of endogenous noncoding RNA that exhibit a variety of biological functions. However, it is not clear whether they are involved in foot-and-mouth disease virus (FMDV) infection and host response. In this study, we established circRNA expression profiles in FMDV-infected PK-15 cells using RNA-seq (RNA-sequencing) technology analysis. The biological function of the differentially expressed circRNAs was determined by protein interaction network, Gene Ontology (GO), and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment. We found 1100 differentially expressed circRNAs (675 downregulated and 425 upregulated) which were involved in various biological processes such as protein ubiquitination modification, cell cycle regulation, RNA transport, and autophagy. We also found that circRNAs identified after FMDV infection may be involved in the host cell immune response. RNA-Seq results were validated by circRNAs qRT-PCR. In this study, we analyzed for the first time circRNAs expression profile and the biological function of these genes after FMDV infection of host cells. The results provide new insights into the interactions between FMDV and host cells.

Keywords: FMDV, RNA-Seq, PK-15 cells, circRNA, differential expression, analysis

Introduction
Circular RNAs (circRNAs) belong to a class of non-coding RNAs that are widespread in the cytoplasm of eukaryotic cells and are structurally and functionally different from linear RNA molecules [1]. They are covalently closed-loop RNA molecules that are formed by back-splicing of the 5′ and 3′ ends of the primary transcript. They have a strong structural stability, tissue, and spatiotemporal specificity [2, 3]. To date, three types of circRNA molecules have been reported including circRNAs generated by reverse exon splicing, circRNAs that form by intronic lasso, and circRNAs consisting of both introns and exons [4–7]. Previously, circRNAs were considered by-products of abnormal splicing during transcription; however, with the rapid development of high-throughput sequencing technologies and bioinformatics, there is growing evidence that circRNAs are involved in regulating a variety of important physiological functions [8]. Most circRNAs are composed of exons and are located in the cytoplasm, indicating that they function as protein regulators in the translation and modification of proteins [7, 9–12]. Recently, it has been shown that circRNAs act as sponges for microRNAs (miRNAs), which act as competing endogenous RNAs...
(ceRNAs) to regulate post-transcriptional gene expression events [13–15]. Other studies have found that circRNAs play important regulatory roles in pathological processes such as neurological diseases [16] and cancer [17, 18]. In addition, because of the low molecular weight of circRNAs, they are transported by extracellular vesicles, such as nanoparticles and exosomes. They are now widely studied and considered as molecular markers, therapeutic targets, and drug carriers in a variety of diseases [19].

As novel regulatory molecules, circRNAs mediate the regulation of viral infections and the cellular immune response, which provide a new perspective for understanding virus–cell interactions. Recently, several studies have shown that circRNAs are also involved in the regulation of virus–host cell interactions as well as in the antiviral cell immune response [5, 20]. For example, during H1N1 influenza A virus (IAV) infection, circGATA2A overexpression in the host promotes replication of the H1N1 IAV virus [21]. Hepatitis C virus infection induces host circRNAs to exert nonsense-mediated decay and inhibit viral replication [22]. Viruses can also use circRNAs to interfere with the host antiviral immune response and help to escape immune surveillance and antiviral immunity. Interestingly, when viral invasion occurs, some circRNAs inhibit immune cell activation; however, when circRNAs are degraded by RNase L, they subsequently regulate autoimmune disease or viral infection clearance by activating PKR activation and downstream cascade responses [23]. Although studies have demonstrated that circRNAs are involved in the regulation of host cells after viral infection, there is a lot to unravel to understand the role that circRNAs play in virus–host interactions and viral pathogenesis.

Foot-and-mouth disease (FMD) is a highly contagious viral disease that occurs in livestock worldwide. FMD is caused by the foot-and-mouth disease virus (FMDV), which mainly infects cloven-hooved animals [24]. Pathological blisters appear in the oral mucosa, extremities, and breasts as the main clinical symptoms, and animals may die from severe infections. This causes significant losses to the animal husbandry industry and the economy [25]. FMDV has a genome of approximately 8.5 kb and is a single-stranded positive-sense RNA virus of the genus Aphthovirus, within the family Picornaviridae. It contains an open reading frame (ORF) that encodes four structural proteins (VP1, VP2, VP3, and VP4) and 10 nonstructural proteins (Lpo, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D) [26–28]. FMDV is divided into seven serotypes (A, O, C, SAT 1, SAT 2, SAT 3, and Asia 1) according to geographical distribution and each serotype has a wide range of antigenic characteristics which do not elicit effective cross-protection. The high incidence and extensiveness worldwide make the prevention and control of FMD a great challenge [29]. Several studies have shown that FMDV is able to escape the host innate immune response through multiple pathways. For example, FMDV antagonizes host antiviral interferon production by inducing PERK and AKT-MTOR signaling to regulate autophagy [30, 31]. FMDV Lpo is able to target ISG15 by specifically cleaving the peptide bonds before the C-terminal Gly-Gly motif. This disrupts the ubiquitination modification process and prevents it from recognizing viral proteins, thereby preventing the initiation of innate immune response signals [32]. FMDV also negatively regulates the activation of the IFN-β signaling pathway by inhibiting the phosphorylation of IRF3 and nuclear translocation through the VP1 protein [33].

Host circRNAs expression and potential role during FMDV infection are unknown. Pigs are the primary natural reservoir of FMDV and have been used as a challenge model [34]. In this study, we used RNA-Seq technology with Illumina HiSeq platform to analyze the characteristics of differentially expressed circRNAs and the biological function of parental genes in PK-15 cells after FMDV infection. RNA-seq results was verified by qRT-PCR. The results provide new clues for understanding the interaction between FMDV and host cells from the perspective of circRNAs.

Materials and methods

Cell culture and virus infection

Porcine kidney cells (PK-15 cells) were cultured at 37 °C in a 5% CO2 humidified atmosphere in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic/antimycotic solution (100U/mL penicillin, 100 μg/mL streptomycin). FMDV serotype A (FMDV A/GD/MM) was provided and stored by the OIE/National Foot and Mouth Disease Reference Laboratory (Lanzhou, Gansu, China). To analyze cell response to FMDV infection, 80% confluent PK-15 cells were inoculated with FMDV or mock infected. After 8 h of infection, both the cell supernatant and precipitate were used for further analysis. All the virus-related experiments were conducted in the Biosafety Level-3 (BSL-3) laboratory of Lanzhou Veterinary Research Institute according to the standard protocols and biosafety regulations provided by the Institutional Biosafety Committee.

RNA extraction, library construction, and RNA-sequencing

Total RNA was extracted from both FMDV-infected and mock-infected cells using TRIzol reagent (Invitrogen, USA). Total RNA was quantified and the quality assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies,
USA), and 1 µg total RNA with a RIN value above 8 was used for library preparation. Pair-End index libraries were constructed according to the manufacturer’s protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). The libraries with human circRNAs sequencing was multiplexed and loaded onto an Illumina HiSeq instrument (Illumina, USA) according to the manufacturer’s instructions. RNA-seq was performed using an Illumina HiSeq to get the raw data (raw reads). Sequencing was carried out in a 2 × 150 paired-end (PE) configuration. All sequences were processed and analyzed by the Shanghai Yuanxin Biomedical Technology Company.

circRNA sequence prediction
After the libraries were sequenced, the PE reads for each sample were mapped to a reference genome (GRCh38, Ensemble 91) using TopHat2 [35, 36].

Differential expression analysis of circRNAs
DE circRNAs were identified and analyzed using DESeq software based on a negative binomial distribution [37, 38]. Differentially expressed (DE) genes were detected with |log₂ (fold change) | ≥ 1 and P value ≤ 0.05.

Gene Ontology and Kyoto Encyclopedia of Gene and Genome pathway analysis
Gene Ontology (GO) analysis was used to annotate the biological processes involved and the functions associated with the parental genes of the circRNAs (molecular functions, cellular components, and biological processes) [39–41]. Kyoto Encyclopedia of Gene and Genome (KEGG) enrichment using hypergeometric test was also conducted to predict the involvement of cellular pathways targeted by circRNAs during FMDV infection [42]. The GO and KEGG pathways identified with corrected P values ≤ 0.05 were considered significantly enriched.

Protein interaction map of parental genes of differentially expressed circRNAs
Protein function related network analysis of circRNAs genes with significant differential expression was done using the Retrieval of Interacting Genes/Proteins (STRING v10) database and web tool [43]. The DE genes were screened with |log₂ (fold change) | ≥ 2 and P value ≤ 0.05.

Differentially expressed circRNAs preparation and qRT-PCR analysis
To validate the accuracy of the sequencing results and screen the partially differentially expressed circRNAs, we designed divergence primers based on the predicted sequences of the circRNAs phenology. The reliability of the sequencing results was verified by RT-qPCR based on selected circRNAs. To remove interference from linear RNA, FMDV infected group samples were treated with Ribonuclease R (RNase R), and the reaction mixture was incubated at 37 °C for 30 min and inactivated in a water bath at 72 °C for 10 min. For the control group, RNA was not treated with RNase R. After that, cDNA was synthesized using reverse transcription kit (Takara, Dalian, China) following the manufacturer’s instructions. The expression of differential circRNAs was verified using the qRT-PCR assay. qRT-PCR was performed at 20 µL reaction volume, including 10 µL SYBR Green Master Mix, 1 µL PCR primers (forward and reverse, respectively), 5 µL nuclease-free water, and 3 µL cDNA. The reaction was performed at 95 °C for 2 min, followed by 45 cycles with 95 °C for 10 s and 60 °C for 10 min. The GAPDH of pigs was used as a reference house-keeping gene. All reactions were run in triplicate. The 2^{- ΔΔCt} method was used to measure expression level of target circRNAs.

Statistical analysis
Statistical significance analysis was performed by the Student’s t-test and P values ≤ 0.05 were considered statistically significant.

Results
Identification and classification of circRNAs
To identify differentially expressed circRNAs in FMDV-infected PK-15 cells, FMDV-infected cells and mock-infected cells were sequenced separately using the Illumina HiSeq platform. As shown in Table 1, a total of 147084824 and 140485672 circRNA raw sequence numbers were identified in FMDV-infected and mock-infected groups, respectively. After filtering and screening, 146011792 and 139140012 high quality sequences were obtained for analysis. Candidate circRNA sequences were mapped to the corresponding genomes for comparison and the FMDV-infected and mock-infected group rates were 87.18% (133109434/152684665) and 91.11%(131008908/143792709), respectively. The Q30 values for the samples were 94.83% and 95.16%. In addition, the circRNA types were identified and characterized by

| Categories          | FMDV infection | NC     |
|---------------------|----------------|--------|
| Raw reads (items)   | 147084824      | 140485672 |
| Clean reads (items) | 146011792      | 139140012 |
| GC content (%)      | 48.71          | 53.26  |
| Q30 (%)             | 94.83          | 95.16  |

Table 1 Summary of Illumina Hiseq sequencing data for circRNAs after infection with FMDV along with the mock group
CIRI2 sequence alignment. Figure 1A shows that the circRNAs in the FMDV-infected and mock-infected groups were mainly derived from exons (85.17%, 87.40%), introns (8.31%, 7.77%), and intergenic regions (6.52%, 4.83%). Circos plots showed the distribution of circRNAs on chromosomes from the FMDV-infected and mock-infected groups (Figure 1B).

**Screening of differentially expressed circRNAs**

With the development of sequencing technologies, high-throughput circRNA sequencing is now a standard method to evaluate circRNAs expression and it is widely used to deduce the effects of host genes in various diseases [44]. There were 1100 differentially expressed circRNAs obtained from PK-15 cells after FMDV infection, of which 425 circRNAs were upregulated and 675 were downregulated. Because of the specificity of circRNAs, SRPBM (Spliced Reads per Billion Mapping) Trans-splicing reads are usually used to estimate circRNA expression. We illustrated the whole expression trend and distribution of all differentially expressed circRNAs using volcano plots (Figure 2A) and clustering heatmaps (Figure 2B). The results showed that FMDV infection of PK-15 cells altered the intracellular circRNAs expression profile, resulting in deregulated expression of multiple circRNAs. This suggests that circRNAs may have a biological function during the cellular response to virus infection.

**Functional analysis of differentially expressed circRNAs**

To further reveal the potential biological functions of FMDV-induced differentially expressed circRNAs, we performed GO and KEGG pathway functional enrichment analysis for selected differentially expressed circRNA genes. GO is a widely used bioinformatics database that includes genes and gene product information for all species [45]. First, GO analysis was performed on differentially expressed circRNAs parental genes. The results showed that differentially expressed circRNAs were mainly involved in nucleic acid metabolism, cellular metabolism, and protein synthesis and modification (Figure 3). It has been demonstrated that circRNAs can actively participate in regulating cellular metabolic processes [46, 47]. Because different genes cooperate with one another to regulate biological processes, further understanding of the potential biological mechanism of differentially expressed circRNAs may be gleaned through KEGG pathway enrichment.
Yang et al. Veterinary Research (2022) 53:24

Analysis. From the figures, it is clear that most of the differentially expressed circRNAs are closely associated with multiple signaling pathways including protein ubiquitination, degradation, cell cycle regulation, RNA transport, autophagy signaling, mTOR signaling, T cell receptor signaling, and nucleotide excision repair (Figure 4). Overall, we found that differentially expressed circRNA genes may regulate multiple pathways, such as nucleic acid metabolism, cellular metabolism, signal transduction, and the immune response to regulate the cellular response to viral infection.

Based on gene function analysis using GO and KEGG (Tables 2 and 3), we explored the regulatory functions of these differentially expressed circRNA genes at the translation level. We screened 64 genes with significantly differentially expressed circRNAs, 8 were significantly upregulated and 56 were downregulated ($p$ value $< 0.05$ and $|\log_2(FC)| > 2$) using the STRING for protein interaction analysis (Figure 5). Interestingly, the results showed that these genes were primarily involved in regulating biological processes such as the protein ubiquitination, cell cycle, and nucleic acid metabolism. In vivo, ubiquitination is a key signaling and defense mechanism for host cells to detect and respond to viral infection, inducing the initiation of immune response signaling pathways against viral invasion. Based on the protein interaction network, circRNAs may play a role in the host immune response induced by FMDV infection.
Figure 3  Top 30 Gene Ontology (GO) functional classifications and enrichment analysis of differentially expressed circRNAs genes. The x-axis indicates the functional description. The y-axis indicates the enrichment significance. GO functional classification from biological process (BP), cellular component (CC), and molecular function (MF).

Figure 4  KEGG classification and pathway enrichment analysis of differentially expressed circRNA genes. (A) KEGG classifications of differentially expressed circRNA genes. The x-axis indicates the number of differentially expressed circRNA genes and the y-axis represents KEGG terms. (B) Top 30 pathway enrichment results for differentially expressed circRNA genes. The x-axis indicates the degree of enrichment, whereas the y-axis represents the functional descriptions of the enriched pathways.
Validation of the differentially expressed circRNAs by qRT-PCR

To verify the reliability of the RNA-seq data, qRT-PCR was performed to detect circRNAs expression change in PK-15 cells infected with FMDV or not. Eight candidate circRNAs were selected to verify their reliability by qRT-PCR. The primers were designed according to the circRNAs position (Table 4). The FMDV infected group was compared to uninfected controls, circ12: 23,921,820–23,923,094, circ6: 37,967,643–37,975,235 were up-regulated. The circ6: 96,667,496–96,668,320, circ2: 64,840,343–64,840,811, circ7: 116,401,757–116,406,130, circ2: 135,356,735–135,368,467, circ6: 85,556,400–85,558,524, circ6: 79,779,444–79,787,224 were significantly down-regulated (Figure 6). These results indicated that the circRNAs in the RNA-seq datasets were reliable.

Discussion

As new rising stars of the noncoding RNA field, circRNAs are a fascinating class of RNAs primarily found in the cytoplasm of eukaryotic cells and are considered to be the product of a reverse-splicing reaction during transcription [48]. Circular RNAs have multiple biological functions and are involved in the regulation of gene expression, disease development, and the immune response [49–51]. However, to date, little is known about the characteristics and functions of circRNA expression in host cells during viral infection. With the development of sequencing technology and bioinformatics, scientists have begun to unravel the roles played by circRNAs in host cells following viral infection. It has been shown that host circRNAs change after infection with certain viruses, such as porcine epidemic diarrhea virus (PEDV) [20], avian leukemia virus (ALV) [5], and transmissible gastroenteritis virus (TGEV) [52]. The function of these differentially expressed circRNAs has been analyzed by GO enrichment, KEGG pathways, and ceRNA networks to elucidate their underlying functions and mechanisms during infection.

FMD is a transmissible disease which rapidly spreads over vast areas and causes devastating effects to the livestock industry. In this study, we infected PK-15 cells with FMDV and identified 1100 differentially expressed circRNAs. Through GO and STING analysis, we found that differentially expressed circRNAs were mainly involved in biological processes, such as host cell nucleic acid metabolism and cell cycle regulation induced by FMDV infection, disruption of the cell cycle, affecting the normal growth status of cells, and oncogenesis. Studies have revealed that circPLK1 promotes breast cancer cell proliferation, migration, and invasion in breast cells [53]. Over-expression of circRNA CCD66 promoted the growth and metastasis of colon cancer cells [54]. In this study, differentially expressed circRNAs, circ12: 23921820–23923094 (KPNB1), circ6: 37967643–37975235 (VPS35), circ2: 64840343–64840811 (DDX39A), and circ7: 116401757–116406130 (DICER1) are involved in biological processes, such as DNA damage repair, nucleic acid metabolism, and transport after FMDV infection. The circRNAs circ6: 96667496–96668320 (CEP192), circ6: 85556400–85558524 (RCC1), circRNA7: 39403319–39407636 (CDC5L) are involved in the regulation of the cell cycle. The above results demonstrate that the differentially expressed circRNAs in this study may be involved in the host response to FMDV infection, but the detailed functions and regulatory mechanisms of these circRNAs need to be further explored.

In addition, according to KEGG functional enrichment, most differentially expressed circRNAs were involved in biological processes such as protein ubiquitination degradation, autophagy, mTOR signaling, and cell cycle regulation, which have a close association with the immune response. Ubiquitination is a key signaling mechanism for host cells to deal with viral infections and includes post-translational modifications and proteolytic reactions. The ubiquitin–proteasome pathway is involved in regulating a variety of cellular processes, such as antigen presentation, cell cycle regulation, apoptosis, immune

Table 2 Differentially expressed upregulated circRNAs (p value < 0.05)

| Circ RNA ID     | Chromosome | Log2FC | Start    | End      | Type | Original gene | Strand |
|----------------|------------|--------|----------|----------|------|---------------|--------|
| 1:205515399–205516988 | 1         | 2.31   | 205515399| 205516988| Exon | CNTLN         | −      |
| 9:45886507–45886672   | 9         | 2.31   | 45886507 | 45886672 | Exon | ARCN1         | +      |
| 11:25838073–25838397  | 11        | 2.16   | 25838073 | 25838397 | Exon | ELL1          | +      |
| 494369484–49406163    | 4         | 2.08   | 94369484 | 94406163 | Exon | ASH1L         | +      |
| 1:90220788–90238411   | 1         | 2.04   | 90220788 | 90238411 | Exon | SENP6         | −      |
| 6:37967643–37975235   | 6         | 2.04   | 37967643 | 37975235 | Exon | VPS35         | +      |
### Table 3  Differentially expressed downregulated circRNAs (p value < 0.05)

| Circ RNA ID | Chromosome | Log2FC | Start   | End     | Type      | Original gene | Strand |
|-------------|------------|--------|---------|---------|-----------|---------------|--------|
| 3.77371816–77378404 | 3 | -2.02 | 77371816 | 77378404 | Exon       | AFTPH        | -      |
| 10:15051106–15056946 | 10 | -2.19 | 15051106 | 15056946 | Exon       | AHCTF1       | +      |
| 7.60719877–60732585 | 7 | -2.65 | 60719877 | 60732585 | Exon       | ARHI1        | -      |
| 13:31573092–31579548 | 13 | -2.46 | 31573092 | 31579548 | Exon       | ARH2         | +      |
| 16:67439729–67445988 | 16 | -2.39 | 67439729 | 67445988 | Exon       | ASC3         | -      |
| 3:107127683–107131029 | 3 | -2.79 | 107127683| 107131029| Exon       | BIRC6        | -      |
| 10:38395600–38396087 | 10 | -2.27 | 38395600 | 38396087 | Exon       | C9orf72      | +      |
| 3.69536711–69540489 | 3 | -2.61 | 69536711 | 69540489 | Exon       | CCT7         | -      |
| 7.39403319–39407636 | 7 | -2.27 | 39403319 | 39407636 | Exon       | CDC5L        | +      |
| 6:96667496–96668320 | 6 | -2.19 | 96667496 | 96668320 | Exon       | CEP192       | -      |
| 9:26111481–26112646 | 9 | -2.34 | 26111481 | 26112646 | Exon       | CEP295       | +      |
| 2.64840343–64840811 | 2 | -2.22 | 64840343 | 64840811 | Exon       | DDX39A       | +      |
| 16:39350968–39516507 | 16 | -2.24 | 39509684 | 39516507 | Exon       | DEPD1C1B     | -      |
| 4:16323825–16335370 | 4 | -2.08 | 16323825 | 16335370 | Exon       | DER1         | -      |
| 7:116401757–116406130 | 7 | -2.11 | 116401757| 116406130| Exon       | Dicer1       | -      |
| 14:11503553–11515969 | 14 | -2.27 | 11503553 | 11515969 | Exon       | ESC02        | +      |
| 7.54870349–54875452 | 7 | -2.19 | 54870349 | 54875452 | Exon       | FANCI        | +      |
| 7.53476795–53477217 | 7 | -2.19 | 53476795 | 53477217 | Exon       | FES          | +      |
| 15:133280528–133283709 | 15 | -2.46 | 133280528| 133283709| Exon       | GIGYF2       | +      |
| 2.143406688–142408143 | 2 | -2.16 | 142406688| 142408143| Exon       | HARS2        | +      |
| 1:129601956–129602162 | 1 | -2.64 | 129601956| 129602162| Exon       | MBD1         | -      |
| 13:200017893–200023406 | 13 | -2.14 | 200017893| 200023406| Exon       | MORC3        | +      |
| 17:48239458–48241671 | 17 | -2.61 | 48239458 | 48241671 | Exon       | NCOAS        | +      |
| 12:40037309–40038184 | 12 | -2.16 | 40037309 | 40038184 | Exon       | NLE1         | +      |
| 12:23859739–23869452 | 12 | -2.34 | 23859739 | 23869452 | Exon       | NPEP5        | +      |
| 5:32992793–3300625 | 5 | -2.52 | 32992793 | 3300625  | Exon       | NUP107       | +      |
| 1:13001290–130015941 | 1 | -2.34 | 13001290 | 130015941| Exon       | NUSA01       | -      |
| 5:313664–341527 | 5 | -2.27 | 313664   | 341527   | Exon       | PPP6R2       | -      |
| 7.28373900–28381978 | 7 | -2.19 | 28373900 | 28381978 | Exon       | PRIM2        | +      |
| 6:87615275–87630082 | 6 | -2.63 | 87615275 | 87630082 | Exon       | PUM1         | -      |
| 4:77612564–77618720 | 4 | -2.07 | 77612564 | 77618720 | Exon       | RB1CC1       | +      |
| 6:85556400–85558524 | 6 | -2.02 | 85556400 | 85558524 | Exon       | RCC1         | +      |
| 11:713800–716256 | 11 | -2.30 | 713800   | 716256   | Exon       | ZMYM2        | -      |
| 3:1137601–11380582 | 3 | -2.34 | 1137601  | 11380582 | Exon       | RFC2         | -      |
| 5:76819452–76857881 | 5 | -2.02 | 76819452 | 76857881 | Exon       | SCAF1        | -      |
| 14:14780708–14801436 | 14 | -2.21 | 14780708 | 14801436 | Exon       | SCML2        | -      |
| 4:74305210–74307728 | 4 | -2.55 | 74305210 | 74307728 | Exon       | SDCCB3       | -      |
| 10:16216508–16239910 | 10 | -2.02 | 16216508 | 16239910 | Exon       | SDCCAG8      | +      |
| 16:21482127–21485938 | 16 | -2.30 | 21482127 | 21485938 | Exon       | SKP2         | +      |
| 15:50832070–50838859 | 15 | -2.02 | 50832070 | 50838859 | Exon       | SMAF1        | +      |
| 3:121012935–121025630 | 3 | -2.16 | 121012935| 121025630| Exon       | SMG6         | +      |
| 6:87847020–87874230 | 6 | -2.41 | 87847020 | 87874230 | Exon       | SNRP400      | -      |
| 12:44919705–44920437 | 12 | -2.14 | 44919705 | 44920437 | Exon       | SPT6H        | +      |
Table 3 (continued)

| Circ RNA ID               | Chromosome | Log$_2$FC | Start   | End     | Type   | Original gene | Strand |
|--------------------------|------------|-----------|---------|---------|---------|---------------|--------|
| 12:42993597–42998085     | 12         | −2.19     | 42993597| 42998085| Exon    | SUZ12         | −      |
| 18:44873297–44897643     | 18         | −2.41     | 44873297| 44897643| Exon    | TAX1BP1       | −      |
| 17:62094151–62114034     | 17         | −2.19     | 62094151| 62114034| Exon    | TCFL5         | −      |
| 16:49107259–49112899     | 16         | −2.17     | 49107259| 49112899| Exon    | TNPO1         | +      |
| 3:106854519–106888705    | 3          | −2.19     | 106854519| 106888705| Exon    | TTC27         | −      |
| 6:79779444–79787224      | 6          | −2.21     | 79779444| 79787224| Exon    | USP48         | −      |
| AEMK02000452.1:1423806–1456508 | AEMK02000452.1 | −2.09     | 1423806 | 1456508 | Exon    | RCOR1         | +      |

Figure 5 Protein function interaction network diagram of differentially expressed circRNA genes. These genes are involved in various biological processes including nucleic acid metabolism, protein ubiquitination modifications, and cell cycle regulation.
responses, inflammation, and viral infections [55]. Various ubiquitination signals play an important role in the activation of the innate immune response. It has been shown that circRNAs participate in the protein ubiquitination process after viral infection. FMDV was able to disrupt the ubiquitination of proteins through multiple pathways and evade killing by the host immune system. For example, FMDV L\textsuperscript{pro} could target and remove the ubiquitin-like protein, ISG15, and specifically cleave peptide bonds at the C-terminal Gly-Gly sequence to disrupt the ubiquitination modification system and inhibit the initiation of the innate immune response [32]. Orf is a worldwide zoonotic disease caused by Orf virus (ORFV). Following ORFV infection, circRNA regulates the ubiquitination process of host cells and affect the host immune response [56]. In this study, differentially expressed circRNA: 79779444–79787224 (USP48) recognized and hydrolyzed the peptide bond at the C-terminal Gly residue of the peptide bonds at the C-terminal Gly-Gly sequence to disrupt the ubiquitination modification system and inhibit the initiation of the innate immune response [32]. Orf is a worldwide zoonotic disease caused by Orf virus (ORFV). Following ORFV infection, circRNA regulates the ubiquitination process of host cells and affect the host immune response [56]. In this study, differentially expressed circRNA: 79779444–79787224 (USP48) recognized and hydrolyzed the peptide bond at the C-terminal Gly residue of the
present study, the circ2: 135356735–135368467 parent-
spatiotemporal specificity [2, 3, 61]. Interestingly, we
pre-mRNA and exhibit strong structural stability and
circRNAs seen in the RNA-seq datasets was reliable.
These results indicated that the differential expression of
a similar expression pattern compared with RNA-seq.
results by circRNAs qRT-PCR. The obtained data showed
ing FMDV infection. We further validated the RNA-Seq
important roles in regulating organism homeostasis dur-
the above functions showed that circRNAs play
viral infection following FMDV infection. The analysis
be involved in FMDV infection-mediated autophagy to
expressed circRNAs in host cells may play an important
strategy for the treatment of Alzheimer’s disease [57].
Other studies have shown that viruses can promote their
own replication by inhibiting cellular autophagy [58].
FMDV also mediates autophagy to evade innate immu-
nity and promote viral replication through multiple path-
ways. FMDV inhibits interferon production by inducing
PERK-mediated autophagy [30]. FMDV VP2 can also
interact with HSPB1 (Heat shock protein family B1) and
activate the EIF2S1-ATF4 pathway to inhibit the AKT-
mTOR signaling pathway, which inhibits autophagy in
host cells to promote viral replication [31]. Heat stress
stimulation can induce the synthesis of heat shock pro-
teins which can repair the damaged proteins and degrade
the unreparable proteins as “chaperones,” maintain
the stable conformation of proteins, ensure the correct
folding of nascent proteins, protect cells against stress
damage, enhance the tolerance of cells, and support the
normal functional metabolism of cells [59, 60]. In the
present study, the circ2: 135356735–135368467 parent-
ral gene, HSPA4, belongs to a member of the heat shock
protein family, which was significantly downregulated.
We hypothesize that circ2: 135356735–135368467 may
be involved in FMDV infection-mediated autophagy to
evade the host immune response. Therefore, differentially
expressed circRNAs in host cells may play an important
role in regulating the immune response mediated by
viral infection following FMDV infection. The analysis
based on the above functions showed that circRNAs play
important roles in regulating organism homeostasis dur-
during FMDV infection. We further validated the RNA-Seq
results by circRNAs qRT-PCR. The obtained data showed
a similar expression pattern compared with RNA-seq.
These results indicated that the differential expression of
circRNAs seen in the RNA-seq datasets was reliable.
CircRNAs are usually generated by back-splicing of
pre-mRNA and exhibit strong structural stability and
spatiotemporal specificity [2, 3, 61]. Interestingly, we
found that a single gene locus could be used to generate
one or more circRNAs through alternative splicing. Our
results show that multiple circRNA subtypes derived
from the same genes were differentially expressed after
FMDV infection in PK-15 cells. These circRNA parental
genes play an important role in regulating the spatiotem-
poral expression of circRNAs.

The regulation of miRNA target gene expression by cir-
cRNAs as miRNA sponges is a classical mechanism of the
cRNAs hypothesis [62, 63]. Most circRNAs act as miR-
NA sponges to regulate gene expression [7, 17]. ciRS-7
functions as a miR-7 sponge and contains more than 70
miRNA binding targets that inhibit miR-7 activity and
promote miR-7 target gene expression [64]. The develop-
ment of metastases in pancreatic cancer was associated
with ciRS-7 regulating miR-7-mediated EGFR/STAT3
signaling [65]. During ORFv infection, circRNAs act as
miRNA sponges to generate circRNA-miRNA-mRNA
networks that indirectly regulate gene expression fol-
lowing ORFV infection [56]. CircRNAs have also been
shown to have essential regulatory roles in colon cancer
[54], breast cancer [66], and liver cancer [67]. In addi-
tion, because of the unique mode of action of circRNAs,
which are stable and enriched in cells, they may be useful
as molecular markers for the diagnosis of ALV and HBV
infections [5, 68].

In this study, we identified and analyzed the functions
of differentially expressed circRNAs in host cells after
FMDV infection based on GO and KEGG functional
enrichment analysis, but we did not predict the miRNA
target sites and miRNA target genes of the differentially
expressed circRNAs. The detailed functions and regula-
tory mechanisms of these differentially expressed circR-
NAs in viral infection or the cell cycle will be the subject
of subsequent studies.

In summary, we found that FMDV infection resulted in
the differential expression of circRNAs within host cells
based on GO and KEGG functional enrichment analy-
sis. The results suggest that these circRNAs are involved
in the regulation of host immune response processes. In
addition, this study was the first to analyze expression
profiles of differential circRNAs and the biological func-
tion of parental genes derived from FMDV-infected host
cells. This provides new insights for researchers to under-
stand the mechanism underlying FMDV–host interac-
tions from the perspective of circRNAs.

Acknowledgements
The authors would like to thank the anonymous editors and reviewers for
their valuable comments and suggestions that proved the quality of this
manuscript.

Authors’ contributions
Conceptualization: KSZ, HKZ and XTL. Formal analysis: JKY and YW. Funding
acquisition: HKZ and KSZ. Investigation: TZ, YH, HMC, WQY and DSZ. Method-
ology: XGY, XHC and CCS. Writing: JKY and KSZ. All authors read and approved
the final manuscript.
Funding
This work was supported by grants from Natural Science Foundation of Gansu Province (20J5RA582) and National Natural Science Foundation of China (31972684).

Declarations

Competing interests
The authors declare that they have no competing interests.

Received: 10 August 2021 Accepted: 18 November 2021 Published online: 21 March 2022

References

1. Hsu MT, Coca-Prados M (1979) Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. Nature 280:339–340

2. Hentze MW, Preiss T (2013) Circular RNAs: splicing’s enigma variations. EMBO J 32:923–925

3. McClintock S, Jens M, Elefsinioti A, Torti F, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Alexander L, Ullrée Z, Markus L, Christine K, Ferdinand N, Nikolaou R (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495:333–338

4. Zhang Y, Zhang XG, Chen T, Xiong JF, Yin QF, Xing YH, Cui H, Yu Y, Dong H, Qu B, Chen Y, Chen L (2013) Circular intronic long noncoding RNAs. Mol Cell 51:792–806

5. Zhang X, Yan Y, Lei X, Li A, Zhang H, Dai Z, Li X, Chen W, Lin W, Chen F, Ma JY, Xie HM (2017) Circular RNA alterations are involved in resistance to avian leukosis virus subgroup-J-induced tumor formation in chickens. Oncotarget 8:34961–34970

6. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO (2012) Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. PLoS One 7:e30733

7. Danan M, Schwartz S, Edelheit S, Sorek R (2012) Transcriptome-wide discovery of circular RNAs in Archaea. Nucleic Acids Res 40:3131–3142

8. Cocquerelle C, Mascrez B, Hétuin D, Bailleul B (1993) Mis-splicing yields circular RNA molecules. FASEB J 7:155–160

9. Du WW, Yang W, Liu E, Yang Z, Dhalwal P, Yang BB (2016) Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. Nucleic Acids Res 44:2846–2858

10. Du WW, Yang W, Chen Y, Wu ZK, Foster FS, Yang Z, Li X, Yang BB (2017) Foxo3 circular RNA promotes cardiac senescence by mediating multiple factors associated with stress and senescence responses. Eur Heart J 38:1402–1412

11. Liang WC, Wong CW, Liang PP, Shi M, Cao Y, Rao ST, Tsui SK, Wayne MM, Zhang Q, Fu WM (2019) Translation of the circular RNA circ-B-catenin promotes liver cancer cell growth through activation of the Wnt pathway. Genome Biol 20:84

12. Legnini I, Di Tirmo G, Rossi F, Morlando M, Brigandi F, Sthandier O, Fatica A, Santini T, Andronache A, Wade M, Pietro L, Nikolaus R, Irene B (2017) Circ-ZNF609 is a circular RNA that can be translated and functions in the beta interferon signaling pathway. J Virol 93:e00588-19

13. Huang G, Zhu H, Shi Y, Wu W, Cai H, Chen X (2015) cir-ITCH plays an inhibitory role in colorectal cancer by regulating the Wnt/b-catenin pathway. PLoS One 10:e0131225

14. Lukiv WJ (2013) Circular RNA (circRNA) in Alzheimer’s disease (AD). Front Genet 4:307

15. Bachmayr-Heyda A, Reiner AT, Auer K, Sukhbaatar N, Aust S, Bacheletner-Hofmann T, Mesteni I, Grunt TW, Zeilinger R, Pils D (2015) Correlation of circular RNA abundance with proliferation—exemplified with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues. Sci Rep 5:8057

16. Cao S, Wei D, Li X, Zhou J, Li W, Qian Y, Wang Z, Li G, Pan X, Lei D (2017) Novel circular RNA expression profiles reflect progression of patients with hypopharyngeal squamous cell carcinoma. Oncotarget 8:45367–45379

17. Li Y, Zheng Q, Bao C, Li S, Guo W, Zhao J, Chen D, Gu J, He X, Huang S (2015) Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. Cell Res 25:981–984

18. Chen J, Wang H, Jin L, Wang L, Huang X, Chen W, Yan M, Liu G (2019) Profile analysis of circRNAs induced by porcine endemic diarrhea virus infection in porcine intestinal epithelial cells. Virology 527:169–179

19. Yu T, Ding Y, Zhang Y, Liu Y, Li J, Zhou J, Song S, Hu B (2019) Circular RNA GATA02A promotes H111 replication through inhibiting autophagy. Vet Microbiol 231:238–245

20. Chen TC, Tallow-Parr M, Cao QM, Kadener S, Böttcher R, Pérez-Vilaró G, Boonchuen P, Sombroonwiat K, Díez J, Sarnow P (2020) Host-derived circular RNAs display pro viral activities in Hepatitis C virus-infected cells. PLoS Pathog 16:e1008346

21. Liu CX, Li X, Nian F, Jiang S, Gao X, Guo SK, Xue W, Cui Y, Dong D, Ding H, Qu B, Zhou Z, Shen N, Yang L, Chen L (2019) Structure and degradation of circular RNAs regulate PKR activation in innate immunity. Cell 177:865–880.e821

22. Grubman MJ, Bass B (2004) Foot-and-mouth disease. Clin Microbiol Rev 17:465–493

23. Li D, Zhang J, Yang W, He Y, Yu Y, Fu S, Li L, Liu X, Zheng H (2019) Poly (I:C) binding protein 2 interacts with VPS and increases the replication of the foot-and-mouth disease virus. Cell Death Dis 10:5156

24. Jamal SM, Belsham GJ (2013) Foot-and-mouth disease: past, present and future. Vet Res 44:116

25. Mason PW, Grubman MJ, Bass B (2003) Molecular basis of pathogenesis of FMDV. Virus Res 91:39–52

26. Yang B, Zhang X, Zhang D, Hou J, Xu G, Sheng C, Choudhury SM, Zhu Z, Li D, Zhang K, Zheng H (2020) Molecular mechanisms of immune escape for foot-and-mouth disease virus. Pathogens 9:729

27. Paton DJ, Reeve R, Capozzo AV, Ludi A (2019) Estimating the protection afforded by foot-and-mouth disease vaccines in the laboratory. Vaccine 37:5515–5524

28. Ranjitha HB, Ammananthan V, Guleria N, Hosamani M, Sreenivasa BP, Dhanesh RV, Santhoshkumar R, Sagar BKC, Mishra BP, Singh RK, Anikut S, Ravi M, Suresh HB (2020) Foot-and-mouth disease virus induces PERK-mediated autophagy to suppress the antiviral interferon response. J Cell Sci 134:jcs240622

29. Sun P, Zhang S, Qin X, Chang X, Cui X, Li H, Zhang S, Gao H, Wang P, Zhang Z, Luo J, Li Z (2018) Foot-and-mouth disease virus cap protein VP2 activates the cellular E1F2S1-ATF4 pathway and induces autophagy via HSPB1. Autophagy 14:336–346

30. Swatek KN, Aumayr M, Pruneda JN, Visser LJ, Berrymann S, Kueck AF, Geurink PR, Ovaa H, van Kuppeveld FJM, Tuthill TJ, Skern T, Komander D (2018) Irreversible inactivation of IGF1S by a viral leader protease enables alternative infection detection strategies. Proc Natl Acad Sci USA 115:2371–2376

31. Zhang W, Yang F, Zhu Z, Wang Y, Cao W, Wang L, Li L, Mao R, Liu Y, Tian H, Zhang K, Liu X, Ma J, Zheng H (2019) Cellular DNAJA3, a novel VP1-interacting protein, inhibits foot-and-mouth disease virus replication by inducing lysosomal degradation of VP1 and attenuating its antagonistic role in the beta interferon signaling pathway. J Virol 93:e00588-19

32. Cubillos C, de la Torre BG, Jakab A, Clementi G, Borrás E, Bäcena J, Andreu D, Sobrino F, Blanco E (2008) Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. J Virol 82:7223–7230

33. Gao Y, Wang J, Zhao F (2015) CIRP: an efficient and unbiased algorithm for de novo circular RNA identification. Genome Biol 16:4

34. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359

35. Gao Y, Wang J, Zhao F (2015) CIRP: an efficient and unbiased algorithm for de novo circular RNA identification. Genome Biol 16:4

36. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140

37. Ando T, Bowers JE, Ming R, Alam M, Paterson AH (2008) Unraveling ancient hexaploidy through multiply-aligned angiosperm gene maps. Genome Res 18:1944–1954
40. Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 11:R14
41. Lu J, Peatman E, Tang H, Lewis J, Liu Z (2012) Profiling of gene duplication patterns of sequenced teleost genomes: evidence for rapid lineage-specific genome expansion mediated by recent tandem duplications. BMC Genomics 13:246
42. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li CY, Wei L (2011) KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 39:W316-322
43. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP, Kuhn M, Bork P, Jensen LJ, Mering CV (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43:D447-452
44. Wang H, Yang J, Yang J, Fan Z, Yang C (2016) Circular RNAs: Novel rising stars in cardiovascular disease research. Int J Cardiol 202:726–727
45. Huntley RP, Sawford T, Mutuowo-Meullenet P, Shypitsyna A, Bonilla C, Martin MJ, O’Donovan C (2015) The GOA database: gene Ontology annotation updates for 2015. Nucleic Acids Res 43:D1057-1063
46. Granados-Riveron JT, Aquino-Jarquin G (2016) The complexity of the translation ability of circRNAs: Biochim Biophys Acta 1859:1245–1251
47. Zou F, Ding Z, Jiang J, Lu F, Xia X, Ma X (2017) Confirmation and preliminary analysis of circRNAs potentially involved in human intervertebral disc degeneration. Mol Med Rep 16:9173–9180
48. Liu M, Bechara FG, Gambichler T, Sand D, Bromba M, Hahn SA, Stockfleth E, Hessam S (2016) Circular RNA expression in cutaneous squamous cell carcinoma. J Dermatol Sci 83:210–218
49. Li X, Liu CX, Xue W, Zhang Y, Jiang S, Yin QF, Wei J, Yao RW, Yang L, Chen LL (2017) Coordinated circRNA biogenesis and function with NF90/NF110 in viral infection. Mol Cell 67:214-227.e7
50. Peng L, Chen G, Zhu Z, Shen Z, Du C, Zhang R, Su Y, Xie H, Li H, Xu X, Xia Y, Tang W (2019) Correction: Circular RNA ZNF609 functions as a competitive endogenous RNA to regulate AKT3 expression by sponging miR-150-5p in Hirschsprung’s disease. Oncotarget 10:3313-3314
51. Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, Zhong G, Yu B, Hu W, Dai L, Zhu P, Chang Z, Wu Q, Zhao Y, Jia Y, Xu P, Liu H, Shan G (2015) Exon-intron circular RNAs regulate transcription in the nucleus. Nat Struct Mol Biol 22:256–264
52. Ma X, Zhao X, Zhang Z, Guo J, Guan L, Li J, Mi M, Huang Y, Tong D (2018) Differentially expressed non-coding RNAs induced by transmissible gastroenteritis virus potentially regulate inflammation and NF-kB pathway in porcine intestinal epithelial cell line. BMC Genomics 19:757
53. Lin G, Wang S, Zhang X, Wang D (2020) Circular RNA circPLK1 promotes breast cancer cell proliferation, migration and invasion by regulating miR-4500/IGF1 axis. Cancer Cell Int 20:593
54. Hisao KY, Lin YC, Gupta SK, Chang N, Yen L, Sun HS, Tsai SJ (2017) Noncoding effects of circular RNA CCDC66 promote colon cancer growth and metastasis. Cancer Res 77:2339–2350
55. Schulman BA, Harper JW (2009) Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. Nat Rev Mol Cell Biol 10:319–331
56. Pang F, Zhang M, Yang X, Li G, Zhu S, Nie X, Cao R, Yang X, Zhang Z, Huang H, Li B, Wang C, Du L, Wang F (2019) Genome-wide analysis of circular RNAs in goat skin fibroblast cells in response to Orf virus infection. Peer J 7:e6267
57. Chen D, Guo Y, Qi L, Tang X, Liu Y, Yang X, Hu G, Shuai Q, Yong T, Wang D, Xie Y, Yang BB, Wu Q (2019) Circular RNA NFI-419 enhances autophagy to ameliorate senile dementia by binding Dymatin-1 and Adaptor protein 2 B1 in AD-like mice. Aging 11:1.2002–12031
58. Chiramel AI, Brady NR, Bartenschlager R (2013) Divergent roles of autophagy in virus infection. Cells 283–104
59. Nicol WS, Boshoff A, Ludewig MH, Hennessy F, Jung M, Blatch GL (2006) Approaches to the isolation and characterization of molecular chaperones. Protein Express Purif 46:1–15
60. Steel GJ, Fullerton DM, Tyson JR, Stirling CJ (2004) Coordinated activation of Hsp70 chaperones. Science 303:98–101
61. Barrett SP, Saltzman J (2016) Circular RNAs: analysis, expression and potential functions. Development 143:1838–1847
62. Tautii R, Loretti C, Pandolfi PP (2013) From pseudo-circRNAs to circ-cerRNAs: a tale of cross-talk and competition. Nat Struct Mol Biol 20:541–543
63. Thomson DW, Dinger ME (2016) Endogenous microRNA sponges: evidence and controversy. Nat Rev Genet 17:272–283
64. Hansen TB, Kjems J, Damgaard CK (2013) Circular RNA and miR-7 in cancer. Cancer Res 73:5609–5612
65. Liu L, Liu FB, Huang M, Xie K, Xie QS, Liu CH, Shen MI, Huang Q (2019) Circular RNA ciRS-7 promotes the proliferation and metastasis of pancreatic cancer by regulating miR-7-mediated EGFR/STAT3 signaling pathway. Hepatobiliary Pancreat Dis Int 18:580–586
66. Lu WY (2017) Roles of the circular RNA circ-Foxo3 in breast cancer progression. Cell Cycle 16:589–590
67. Xu L, Zhang M, Zheng X, Yi P, Lan C, Xu M (2017) The circular RNA ciRS-7 (Cdr1as) acts as a risk factor of hepatic microvascular invasion in hepatocellular carcinoma. J Cancer Res Clin Oncol 143:17–27
68. Rao X, Liu L, Li X, Wang L, Li A, Yang Q (2021) N(6)-methyladenosine modification of circular RNA circ-ARL3 facilitates Hepatitis B virus-associated hepatocellular carcinoma via sponging miR-1305. IUBMB Life 73:408–417

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:
• fast, convenient online submission
• thorough peer review by experienced researchers in your field
• rapid publication on acceptance
• support for research data, including large and complex data types
• gold Open Access which fosters wider collaboration and increased citations
• maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions