Identification and characterization of microRNA in the lung tissue of pigs with different susceptibilities to PCV2 infection

Ping Zhang1†, Liyuan Wang1†, Yanping Li1, Ping Jiang2, Yanchao Wang1, Pengfei Wang1, Li Kang1, Yuding Wang1, Yi Sun1* and Yunliang Jiang1*

Abstract
Porcine circovirus type 2 (PCV2) is the primary cause of post-weaning multisystemic wasting syndrome (PMWS) and other PCV-associated diseases. According to our previous RNA-sequencing analysis, the differences in the susceptibility to PCV2 infection depended on the genetic differences between the Laiwu (LW) and Yorkshire × Landrace crossbred (YL) pigs, but the cellular microRNA (miRNA) that are differentially expressed between the LW and YL pigs before and after PCV2 infection remain to be determined. In this study, high-throughput sequencing was performed to determine the abundance and differential expression of miRNA in lung tissues from PCV2-infected and PCV2-uninfected LW and YL pigs. In total, 295 known and 95 novel miRNA were identified, and 23 known and 25 novel miRNA were significantly differentially expressed in the PCV2-infected vs. PCV2-uninfected LW pigs and/or the PCV2-infected vs. PCV2-uninfected YL pigs. The expression levels of ssc-miR-122, ssc-miR-192, ssc-miR-451, ssc-miR-486, and ssc-miR-504 were confirmed by quantitative real-time PCR (qRT-PCR). Analysis of the potential targets of the four up-regulated miRNA (i.e., ssc-miR-122, ssc-miR-192, ssc-miR-451 and ssc-miR-486) identified pathways and genes that may be important for disease resistance. Among the up-regulated miRNA, ssc-miR-122 can repress the protein expression and viral DNA replication of PCV2 and down-regulate the expression of the nuclear factor of activated T-cells 5 (NFAT5) and aminopeptidase puromycin sensitive (NPEPPS) by binding to their 3′ untranslated region (3′UTR) in PK15 cells. Therefore, ssc-miR-122 may indirectly suppress PCV2 infection by targeting genes related to the host immune system, such as NFAT5 and NPEPPS.

Introduction
Porcine circovirus (PCV) was first considered a contaminant in a porcine kidney cell line in 1974 and was described in greater detail in 1982. In the late 1990s, PCV2 was found to be associated with post-weaning multisystemic wasting syndrome (PMWS), which is currently considered one of the most important swine diseases worldwide [1]. PCV2 belongs to the Circoviridae family, which is characterized by a genome consisting of single-strand circular DNA with 1768-9 nucleotides (nt) [2, 3]. PCV2-affected pigs show wasting and progressive weight loss, enlarged lymph nodes, and respiratory distress, jaundice, and occasional diarrhea [4]. Morbidity in PCV2-affected farms is commonly 4–30% (occasionally as high as 50–60%), and mortality ranges from 4 to 20% [5]. Because of the increased mortality rates and the impact on weight gain, PCV2 has had a serious economic impact on the swine production industry [6]. The replication patterns of PCV2 in pulmonary alveolar macrophages are different among macrophages derived from different conventional crossbred pigs [7].

MicroRNA (miRNA) are ~21–23 nt small RNA (sRNA) molecules that regulate gene expression at the post-transcriptional level [8]. Currently, miRNA have been estimated to constitute 1–5% of animal genes and collectively regulate up to 30% of genes; therefore, miRNA...
are among the most abundant regulators [9]. No miRNA encoded by PCV2 genomic DNA were detected in tonsil and mediastinal lymph node tissues from PCV2-infected pigs [10]. In PK15 cells expressing PCV2 ORF1, ORF2 and ORF3, 51, 74 and 32 miRNA were identified, respectively, differing in abundance from those in the controls [11]. In PCV2-infected Landrace × Yorkshire pigs, miR-126-3p, 126-5p, 129a, let-7d-3p and let-7b-3p were up-regulated, while miR-193a-5p, 574-5p and 34a were down-regulated in the mediastinal lymph node [12]. These differentially expressed miRNA are mainly involved in the regulation of the immune system and cell proliferation. Although certain PCV2 infection-associated miRNA have been identified, pig-breed-specific miRNA that are differentially expressed and likely account for the resistance to PCV2 infection have not been characterized.

The Laiwu (LW) pig is a Chinese indigenous pig breed from Shandong Province that is well-known for its extremely high intramuscular fat content of > 10%. The LW pig also exhibits a higher resistance to certain infectious diseases, including PCV2. In our previous study, PCV2-infected Yorkshire × Landrace (YL) pigs exhibited serious clinical features that are typical of PCV2 disease, particularly severe lesions in the lungs, such as congestion, bleeding, interstitial pneumonia and lymphocyte infiltration, while the PCV2-infected LW pigs showed only a few clinical symptoms; at 35 days post-infection (dpi), the PCV2 DNA copy in the YL pigs was significantly higher than that in the LW pigs [13]. In this study, using Illumina/Solexa high-throughput sequencing, we identified the differentially expressed miRNA in the lung tissues between LW and YL pigs prior to and post PCV2 infection and further characterized the role of miR-122 in conferring higher resistance to PCV2 infection.

Materials and methods
Sample collection
Fifteen purebred LW and 15 YL weaned pigs that were validated to be free of PCV2, porcine circovirus type 1 (PCV1), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV) were raised under identical conditions on the same farm. All pigs were randomly divided into the following four groups: PCV2-infected LW pigs (LW-i, n = 10), PCV2-uninfected LW pigs (LW-u, n = 5), PCV2-infected YL pigs (YL-i, n = 10) and PCV2-uninfected YL pigs (YL-u, n = 5). The procedures used for the pig management and PCV2 inoculation have been previously described [13, 14]. Briefly, the PCV2 strain (named PCV2-SD) used in this experiment was isolated from suspected PMWS pigs in the Shandong province. The virus genome of PCV2-SD is 1767 bp in size and phylogenetic analysis based on both the whole genome and ORF2 sequences indicate that the genotype of PCV2-SD is PCV2b. Each pig from LW-i and YL-i groups was intramuscularly injected with 3 mL PCV2-SD solution with 10⁵⁵⁵ TCID₅₀ (50% tissue culture infective dose)/mL. The pigs from LW-u and YL-u groups were treated similarly with an identical volume of phosphate buffered saline (PBS). During the experimental period, clinical signs were monitored daily and the copy number of PCV2 DNA in serum was detected by quantitative real-time PCR (qRT-PCR) at 0, 4, 7, 10, 14, 21, 28 and 35 dpi. From 7 dpi, PCV2 virus could be detected from the serums of PCV2-infected pigs, indicating that the intramuscular injection was successful. The mean copy number of PCV2 DNA in serum significantly increased in both PCV2-infected LW and YL pigs at 14 dpi. PCV2-infected YL pigs exhibited serious clinical signs typifying PMWS, while the PCV2-infected LW pigs showed slightly clinical symptoms. The lung tissue of PCV2-infected YL pigs showed serious lesions, while they were not observed in PCV2-infected LW pigs [13]. All pigs were sacrificed at 35 dpi and tissue samples were frozen in liquid nitrogen or preserved by immersion in 10% neutral-buffered formalin.

sRNA library preparation and deep-sequencing
Four sRNA libraries of LW-u, LW-i, YL-u and YL-i were constructed using the homogenized and pooled total RNA from four individuals selected from each group as previously described [15–17]. The construction of pooled small RNA libraries is certainly better than pooled total RNA before Illumina sequencing. To minimize deviation of this procedure compared with the results coming from each individual per group, more miRNA were validated by qRT-PCR in this study. Total RNA was extracted from the lung tissues of PCV2-infected and uninfected pigs of each breed with TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly, for each sample, 20 μg of total RNA and a Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA) were used for library construction according to the manufacturer’s instructions. Then, fractions between 18 and 30 nt were removed and purified using 15% Tris–borate-EDTA denaturing polyacrylamide gel electrophoresis (PAGE). Subsequently, the 3’ and 5’ RNA adaptors were ligated to the purified fragments with T4 RNA ligase in proper order. The cDNA from the adaptor-ligated sRNA were then amplified by RT-PCR with 15 cycles. The products (90-bp sRNA + adaptors) after purification on 4% agarose gels were used for sequencing on an Illumina 1G Genome Analyzer at Beijing Genomics Institute (BGI, Shenzhen, China). After masking and removing the redundant reads, the clean reads were processed for further analysis.
Alignment, annotation, and clustering of reads
The initial output was converted into raw sequence data in a base-calling step. The reads were sorted according to the barcode index, and the adapter sequences were trimmed. The remaining 18–30 nt identical high-quality sequences were counted, and the unique sequences and their associated read counts were mapped to the Sus scrofa genome assembly with no mismatches using SOAP v.1.11 (short oligonucleotide alignment program) [18] to analyze their expression and distribution. The sequences that perfectly matched the reference genome sequence were retained for further analysis. The sequences were aligned against both known miRNA precursors and mature miRNA deposited in miRBase [19] (Release 21) to identify the conserved miRNA. The clean reads were compared with the sRNA deposited in the GenBank and Rfam [20] databases. The sRNA sequences were annotated using the tag2 annotation software developed by BGI. Because some sRNA tags may be mapped to more than one sRNA category, the following priority rule was used to ensure uniqueness: sRNA (GenBank > Rfam) > known miRNA > repeat > exon > intron [21]. The characteristic hairpin structures of the miRNA precursor sequences were used to predict the novel miRNA.

Differential expression analysis and hierarchical clustering of miRNA
To compare the miRNA expression levels between any two samples, the expression of the miRNA in the two series (LW-i vs. LW-u and YL-i vs. YL-u) were normalized to obtain the expression of transcripts per million reads. If the normalized expression of a given miRNA was zero, its expression value was set to 0.01. If the normalized expression of a given miRNA was less than 1 in both samples of a sample pair, the miRNA was removed from the differential expression analysis. The fold-change and P value of each miRNA in each sample pair were calculated using previously published criteria [15–17]. A hierarchical clustering analysis of the miRNA expression levels was performed using PermutMatrix software. The relative expression level of each miRNA was calculated as the total number of reads in the four libraries.

qRT-PCR
Differentially expressed miRNA and target mRNA were validated using qRT-PCR according to the manufacturer’s protocol. Lung tissues from 16 pigs (four groups with n = 4 per group) sequenced by BGI were used as substrates for the qRT-PCR, which was performed using an Mx3000p™ SYBR Green qRT-PCR Analyzer (Stratagene, CA, USA).

For the miRNA detection, the SYBR®PrimeScript™ miRNA RT-PCR Kit (Takara Biotechnology Co., Ltd., Japan) was used. The reverse transcription system included 10 μL of 2 × miRNA reaction buffer, 2 μL of 0.1% BSA, 2 μL of miRNA PrimeScript™ RT Enzyme Mix, 2 μL of total RNA (1 mg/mL), and up to 20 μL RNase-free H2O. The reverse transcription program was as follows: 37 °C for 60 min, followed by 85 °C for 5 s. The cDNA was then used for the real-time PCR quantification of the miRNA using a miRNA-specific primer and the Uni-miR qPCR primer developed by Takara. 5S rRNA was used as an endogenous control as listed in Table 1. The real-time PCR reaction mixture was prepared on ice and comprised 10 μL of 2 × SYBR® Premix Ex Taq™ II, 0.8 μL of PCR forward primer (10 μM), 0.8 μL of Uni-miR qPCR primer (10 μM), 0.4 μL of 50 × ROX reference dye II, 2 μL of cDNA, and up to 20 μL of H2O. Standard curves with threefold dilutions (from a pool consisting of 16 cDNA samples) were generated for each assay, and the amplification efficiency was calculated based on the slopes of the standard curves. The reaction mixtures were incubated in a 96-well plate at 95 °C for 2 min, followed by 45 cycles of 95 °C for 20 s, 60 °C for 35 s and 72 °C for 15 s. The Mx3000/Mx Pro software (Stratagene) was used to construct the melting curve. All reactions were performed in triplicate.

For the mRNA, the qRT-PCR was performed using the TaKaRa PrimeScript™ RT Reagent Kit and gDNA Eraser (TaKaRa), which comprised a genomic DNA elimination reaction, reverse-transcription reaction and RT-PCR. The PCR primers targeted the exon/exon junctions using DNAMAN 6.2 to avoid possible amplification of any residual genomic DNA, and the specificity was determined using BLASTN. The GAPDH gene was used as the internal control, and all primer sequences are shown in Table 1. A melting curve confirmed the specificity of

| miRNA/mRNA | Primers |
|------------|---------|
| ssc-mir-122 | GTGGAGTGTGCAATGTTTGA |
| ssc-mir-451 | CGAGGAAACGTTACATTAGTGGT |
| ssc-mir-504 | GGAAGCCCTTGCCATCTCTATCT |
| ssc-mir-486 | CCGTAGTACGGCTCGCCG |
| ssc-mir-192 | CCTGACCATAGAAGACGCAAA |
| sSRNA | GTTACTCTGAGTGCTGCC |
| GAPDH-F | TCTTCGCTGAGTAGGAGACTG |
| GAPDH-R | GTTTCGATGGGGGCAGT |
| NFAF5-T | AGAGTAGTGGTGCTGCTG |
| NFAF5-R | CAGGGAGTGTGTTACCG |
| NFEPPS-F | AGATGGTGTTTGGTCC |
| NFEPPS-R | CGTGATGAAGAACAGGAGT |
each product, and PCR analyses were performed in triplicate in 20 μL amplification reactions containing 10 μL of 2 × SYBR® Premix Ex Taq™ II, 0.4 μL of ROX II, 2 μL of RT reaction solution (cDNA solution), 6 μL of H2O and 0.8 μL (10 mM) of each primer using the following conditions according to the manufacturer’s instructions: 1 cycle of 95 °C for 3 min, 40 cycles of 95 °C for 20 s, 58–61 °C for 20 s and then 72 °C for 15 s. A melting curve analysis (60–95 °C) was performed to assess the amplification specificity.

The relative expression levels of each mRNA and miRNA were calculated using the 2–ΔΔCT method according to the standard curve [22]. Each sample was replicated three times. The levels are expressed as the 2–ΔΔCT means ± standard errors (SEs).

miRNA target prediction and bioinformatic analysis

The bioinformatic prediction of the miRNA targets was performed using TargetScan Release 6.2 [23]. Due to no database for porcine miRNA in the TargetScan Release 6.2, so the prediction was done using human orthologs of porcine miRNA, assuming that humans and pigs have conserved miRNA-targeting sites at the 3′ UTRs of orthologous mRNA. A functional clustering analysis of the identified miRNA was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database and DIANA-miRPath [24]. Four of the validated up-regulated miRNA (i.e., ssc-miR-122, ssc-miR-192, ssc-miR-451 and ssc-miR-486) were selected as the input miRNA for the target gene prediction.

Cell culture and transfection

PK15 cells were maintained in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco) in a humidified atmosphere of 5% CO2 at 37 °C. The PK15 cells were transfected with the ssc-miR-122 mimic and a negative control mimic using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfection complexes were added to the medium at a final oligonucleotide concentration of 30 nM. Similarly, the PK15 cells were transfected with the ssc-miR-122 inhibitor or inhibitor negative control at a final oligonucleotide concentration of 100 nM to knockdown ssc-miR-122. The culture medium was replaced 6 h post-transfection with the regular culture medium for 24 h. The miRNA transfection efficiency was verified by qRT-PCR.

Luciferase reporter assay

To evaluate the interaction between miR-122 and the target genes nuclear factor of activated T-cells 5 (NFAT5) and aminopeptidase puromycin sensitive (NPEPPS), the 3′-untranslated region (3′UTR) sequence of NFAT5 and NPEPPS was constructed into the pGL3-promoter vector (Promega, WI, USA) using the XbaI restriction sites. The recombinant plasmids were named NFAT5 3′UTR WT and NPEPPS 3′UTR WT (Figure 5D). The NFAT5 and NPEPPS 3′UTR sequence complementary to the ssc-miR-122 seed sequence (GUGA) was then mutated to TGAG and TGAC, and the mutated plasmids were named NFAT5 3′UTR MT and NPEPPS 3′UTR MT (Figure 5D). All above plasmids were confirmed by sequencing. The cells were co-transfected with the reporter construct, an internal control vector (pGL4.74), and a synthetic ssc-miR-122 or negative control mimic. Forty-eight hours after the transfection, the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The relative luciferase expression values were analyzed using a Modulus single-tube multimode reader (Turner BioSystems, CA, USA).

Western blotting

The total protein was extracted from the PK15 cells using cell lysis buffer for Western blotting (Beyotime, China) with PMSF (Beyotime). The protein concentration of the cell lysate was quantified using a BCA kit (Beyotime), and 40 μg of each protein sample were separated on SDS-PAGE 8% gels and blotted onto a polyvinylidene fluoride (PVFD) membrane (Millipore, USA). The membranes were blocked for 2 h at room temperature in Western Blocking Buffer (Beyotime) and then incubated overnight at 4 °C with rabbit polyclonal antibodies against NFAT5 and NPEPPS (Abcam, USA) at a dilution of 1:1000. To normalize the protein loading, the PVDF membranes were simultaneously incubated with the mouse anti-GAPDH (Beyotime) monoclonal antibody at a dilution of 1:1000. The membranes were washed three times with TBST buffer and then incubated with HRP-conjugated secondary antibodies that were diluted 1000 times at room temperature for 1 h. Finally, the immunoreactive bands were visualized using a DAB Horseradish Peroxidase Color Development Kit (Beyotime).

Quantification of viral DNA

To determine the effect of ssc-miR-122 on viral protein expression and viral replication, PK15 cells were pre-transfected with either ssc-miR-122 mimic (30 nM) or mimic control and then infected with PCV2-SD at a multiplicity of infection (MOI) of 0.1 TCID50. At 36 h post-infection, the expression levels of the PCV2 Cap protein were analyzed by Western blotting using mouse anti-Cap monoclonal IgG, and the numbers of PCV2 DNA copies were quantified by absolute qRT-PCR, as described below. The viral DNA was extracted from the PK15 cells using a Viral DNA Kit (OMEGA, China) and used for quantifying the copy number of PCV2 genomic DNA by qRT-PCR. The primers (PCV2-RT-F:
5′-CCAGGAGGGCGTTCTGACT-3′ and PCV2-RT-R: 5′-CGTTACCGCTGGAGAAGGAA-3′) were designed according to PCV2-SD sequence. A 99 bp conserved region of the ORF2 gene of PCV2 was amplified by PCR and cloned into a pMD-18T vector (TaKaRa). The resultant plasmid was used as a standard DNA template to optimize the assay conditions. The PCV2 genomic DNA copy number was analyzed by qRT-PCR using the following conditions: 95 °C for 10 s, 95 °C for 5 s, 55 °C for 30 s, 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s for 40 cycles. The baseline adjustment method in the MX3000p software (Stratagene) was used to determine the Ct value of each reaction. The copy number in the sample was measured using a linear formula that was established according to the standard curve using the tenfold serial dilution of the standard plasmid. All samples were amplified in triplicate.

Statistical analysis
The results shown in the figures represent at least three independent trials and are presented as the averages ± SE as indicated. One-way ANOVA and Duncan’s Multiple Range test (SAS version 8.02, 2001) were performed to examine the significance of the differential expression levels among the groups, and the differences among the groups were considered statistically significant at P value < 0.05.

Results
Sequence analysis of sRNA in porcine lung tissues
The four sRNA libraries of LW-u, LW-i, YL-u and YL-i (each from the pooled lung tissues of four individuals) were constructed and sequenced using an Illumina/Sol- exa 1G high-throughput sequencer. In total, 9,573,498 (LW-u), 9,570,066 (LW-i), 9,571,684 (YL-u), and 9,573,660 (YL-i) high-quality reads were obtained. After removing adaptors and insufficient tags, 9,440,912 (LW-u), 9,455,761 (LW-i), 9,511,998 (YL-u), and 9,533,777 (YL-i) clean reads of 18-30 nt were ultimately retained (Table 2). Amongst these reads, 61.04% (LW-u), 65.22% (LW-i), 64.41% (YL-u), and 63.39% (YL-i) of the total reads were perfectly mapped to the Sus scrofa genome (Additional file 1). Most sRNA were 19–24 nt in length. The 22-nt long sRNA sequences were the most abundant and accounted for more than 50% of the sRNA, followed by the 23-nt and 21-nt long sRNA, which is typical of sRNA Dicer-processed products and is consistent with the known 18–25 nt range of miRNA (Additional file 2). To assess the sRNA detection efficiency of the high-throughput sequencing, all sequence reads were annotated and classified by aligning the sequence reads to sequences in the GenBank and Rfam databases. Consequently, 35,332 (LW-u), 45,265 (LW-i), 48,905 (YL-u), and 53,405 (YL-i) (15.72, 15.95, 16.52, and 16.84%, respectively) unique sRNA were annotated in the four libraries.

Table 2 Summary of clean reads produced by the sRNA sequencing after filtering the contaminated reads

| Type                                      | LW-u          | LW-i          | YL-u          | YL-i          |
|-------------------------------------------|---------------|---------------|---------------|---------------|
| Amount                                    | 9,600,000     | 9,573,498     | 9,571,684     | 9,573,660     |
| Percent (%)                               | 100           | 100           | 100           | 100           |
| High_quality                              | 9,573,498     | 9,570,066     | 9,571,684     | 9,573,660     |
| 3′adapter_null                            | 1308          | 1405          | 1626          | 1307          |
| 0.01                                      | 0.01          | 0.01          | 0.02          | 0.01          |
| Insert_null                               | 2740          | 2833          | 1254          | 1075          |
| 0.03                                      | 0.03          | 0.03          | 0.01          | 0.01          |
| 5′adapter_contaminants                    | 108,280       | 71,737        | 35,436        | 26,874        |
| 1.13                                      | 0.75          | 0.37          | 0.28          |
| Smaller_than_18nt                         | 20,206        | 38,263        | 21,318        | 10,545        |
| 0.21                                      | 0.40          | 0.22          | 0.11          |
| PolyA                                     | 52            | 67            | 52            | 82            |
| 0.00                                      | 0.00          | 0.00          | 0.00          |
| Clean_reads                               | 9,440,912     | 9,455,761     | 9,511,998     | 9,533,777     |
| 98.62                                     | 98.81         | 99.38         | 99.58         |

- **Total_reads**: total sequenced reads, which are generally required to be > 5 million.
- **High_quality**: number of high-quality reads.
- **3′adapter_null**: number of reads with no 3′ adaptor.
- **Insert_null**: number of reads with no insertion.
- **5′adapter_contaminants**: number of 5′ contaminants.
- **Smaller_than_18nt**: number of reads less than 18 nt.
- **PolyA**: number of reads with polyA.
- **Clean_reads**: number of clean reads after removing the adaptors and contaminants. Clean reads were used in all analyses in this study.

Known conserved and differentially expressed miRNA in porcine lung tissues
The BLASTN searches (number of mismatches ≤ 3) and further sequence analyses showed that 5,404,421 (LW-u), 5,484,886 (LW-i), 5,358,244 (YL-u), and 5,279,602 (YL-i) sRNA sequences perfectly matched known pig, human
Table 3 Ten most abundantly co-expressed miRNA in porcine lung tissues

| Mir-namea | LW-i-stdb | LW-u-stdc | Fold-change | P vauled | Sig-labele | YL-i-std | YL-u-std | Fold-change | P value | Sig-label |
|-----------|-----------|-----------|-------------|-----------|------------|----------|----------|-------------|---------|-----------|
| ssc-let-7a | 200 116.9 | 223 606.8 | −0.160121   | 0         | #          | 190 431.98 | 205 049.8 | −0.106698   | 0       | #         |
| ssc-let-7f | 153 283.9 | 147 584.8 | 0.05466     | 7.91E−22  | #          | 154 279.99 | 162 951.5 | −0.078891   | 0       | #         |
| ssc-let-7c | 23 084.45 | 26 634.82 | −0.206393   | 0         | #          | 25 229.77 | 25 631    | −0.022763   | 4.02E−08 | #         |
| ssc-miR-103 | 19 191.47 | 14 749.21 | 0.37983     | 0         | #          | 27 233.803| 13 713.84 | 0.98977     | 0       | #         |
| ssc-miR-140-3p | 18 098.79 | 15 669.88 | 0.20789     | 0         | #          | 13 130.386| 13 746.64 | −0.066172   | 4.09E−31 | #         |
| ssc-miR-107 | 13 082.61 | 16 618.84 | −0.34517    | 0         | #          | 12 454.455| 13 656.54 | −0.13293    | 1.15E−116| #        |
| ssc-miR-199a-3p | 11 877.63 | 10 953.92 | 0.1168      | 8.59E−79  | #          | 10 987.566| 12 309.09 | −0.163852   | 2.36E−157| #        |
| ssc-miR-199b-3p | 11 877.52 | 10 953.92 | 0.11679     | 8.94E−79  | #          | 10 987.461| 12 308.88 | −0.163841   | 2.48E−157| #        |
| ssc-let-7g | 10 709.87 | 9 836.656 | 0.1227      | 2.92E−78  | #          | 9 316.1999| 10 257.47 | −0.13887    | 8.59E−96  | #        |
| ssc-miR-107 | 10 397.37 | 8 750.532 | 0.24878     | 2.47E−293 | #          | 7 124 6684| 7 155 805 | −0.006291   | 0.421371 | #        |

a Mir-name: miRNA name.
b std: normalized expression level of miRNA in each sample.
c Fold-change = \( \log_2 \) (treatment/control).
d P value: the significance of the differential miRNA expression between the samples; low P values indicating higher significance of miRNA expression.
e Sig-label
f #: no significant difference.
files 4 and 5). In LW pigs, seven novel miRNA were significantly up-regulated ($P < 0.01$; fold-change of $< -1$ or $> 1$) and five novel miRNA were significantly down-regulated after PCV2 infection. In YL pigs, seven novel miRNA were significantly up-regulated, and nine novel miRNA were significantly down-regulated. Of these differentially expressed novel miRNA, novel-miR-25 was up-regulated and novel-mir-60 was down-regulated in both breeds, while the expression of novel-mir-63 changed in the opposite direction in different breeds (Additional file 4).

Validation of differentially expressed miRNA and affected pathways

The qRT-PCR validation of the differentially expressed miRNA indicated that five miRNA (i.e., ssc-miR-122, ssc-miR-192, ssc-miR-451, ssc-miR-486 and ssc-miR-504) had differential expression patterns that were similar to the sRNA sequencing data (Figure 2). Of these miRNA, ssc-miR-122 and ssc-miR-192 had significantly higher expression levels in the PCV2-infected LW pigs than those in the uninfected LW pigs, while the expression of ssc-miR-486 in both breeds of PCV2-infected pigs was significantly higher than that in the uninfected pigs. The TargetScan analyses using these miRNA predicted significantly conserved genes in several KEGG pathways, including the insulin signaling pathway [nine predicted target genes, $-\ln(P value) = 7.75$], the mTOR signaling pathway [four genes, $-\ln(P value) = 4.75$], non-small cell lung cancer (NSCLC) [four genes, $-\ln(P value) = 3.71$], and leukocyte transendothelial migration [six genes, $-\ln(P value) = 3.61$] (Additional file 6).

Expression profile of miR-122 in different tissues

MiR-122 is a broad-range miRNA that can express in many tissues; therefore, we assessed the expression profile of miR-122 in different tissues, including the lymph node, small intestine, tonsil, large intestine, liver, spleen and heart, in the LW and YL pigs after the PCV2 infection. A comparison of the PCV2-infected pigs and PCV2-uninfected pigs indicates that the expression of miR-122 was significantly higher in the hearts of the infected LW pigs ($P < 0.05$) and the livers of the infected YL pigs ($P < 0.01$) (Figure 3). Meanwhile, in both pig breeds, the expression of miR-122 was higher in the tonsil and spleen and lower in the lymph node and small intestine.
Figure 2  Five differentially expressed miRNA in porcine lung tissues were validated by qRT-PCR. The order of miRNA shown from top to bottom is ssc-miR-122, ssc-miR-192, ssc-miR-451, ssc-miR-486 and ssc-miR-504. The Solexa sequencing results are shown on the right, and the qRT-PCR results are shown on the left. * and ** indicate significance at the P-value threshold levels of 0.05 and 0.01, respectively. The expression levels of three of the five miRNA (except for ssc-miR-451 and ssc-miR-504) were consistent with the sequencing results with P < 0.05.
of the infected pigs than that in the uninfected pigs, but this pattern was not statistically significant \((P > 0.05)\) (Figure 3).

**MiR-122 can repress the protein expression and viral DNA replication of PCV2**

As mentioned in materials and methods, PK15 cells were pretransfected with either ssc-miR-122 mimic or mimic control and then infected with PCV2. At 36 h post-infection, the expression levels of the PCV2 Cap protein and the copy numbers of PCV2 DNA were quantified. As shown in Figure 4, the Cap protein expression and viral DNA copies were significantly reduced when the cells were transfected with ssc-miR-122 compared to those in the mimic-control-treated infected cells and untreated infected cells \((P < 0.01)\). Thus, ssc-miR-122 could decrease PCV2 viral DNA replication and protein synthesis in PK15 cells.

**Nuclear factor of activated T-cells 5 (NFAT5) and aminopeptidase puromycin sensitive (NPEPPS) were directly targeted by miR-122**

Since the expression of miR-122 was greatly increased in the LW pigs but remained much lower in the YL pigs after the PCV2 infection, the target genes of miR-122 in PCV2 infection were further analyzed. According to the bioinformatic predictions, miR-122 had a high complementarity and binding specificity with putative binding sites within the 3' UTR of the porcine NFAT5 and NPEPPS genes. To confirm the relationship between miR-122 and the NFAT5 and NPEPPS genes, we first measured the expression levels of NFAT5 and NPEPPS in the lung tissue. NFAT5 was significantly down-regulated in the PCV2-infected pigs compared with that in the uninfected pigs \((P < 0.05)\), which was the opposite of the expression levels of miR-122 (Figure 5A). The changes in the expression of NPEPPS were similar to those observed in NFAT5 (Figure 5A). The transfection of ssc-miR-122 into the PK15 cells markedly decreased the mRNA \((P < 0.01)\) and protein levels of NFAT5 \((P < 0.05)\), and mRNA and protein levels of NPEPPS \((P < 0.05)\) (Figures 5B and C). Next, we verified that NFAT5 and NPEPPS are the direct targets of ssc-miR-122 using the vector pGL3-promoter linked to wild or mutant 3' UTRs of porcine NFAT5 and NPEPPS genes, respectively (Figure 5D). The co-transfection of the luciferase constructs with the ssc-miR-122 mimic into the PK15 cells showed that the ssc-miR-122 mimic significantly decreased the luciferase activity from the luciferase constructs harboring the wildtype 3' UTR of both NFAT5 and NPEPPS but not of the mutant 3'
Figure 5 Validation of NFAT5 and NPEPPS as direct targets of ssc-miR-122  A mRNA expression of NFAT5 and NPEPPS in lung tissues from uninfected or infected LW and YL pigs. B mRNA expression of NFAT5 and NPEPPS in PK15 cells transfected with the ssc-miR-122 mimic. C Protein expression of NFAT5 and NPEPPS in PK15 cells transfected with ssc-miR-122 mimic. D Diagram of the predicted miR-122 targeting site within the 3' UTR of NFAT5 and NPEPPS (3' UTR WT). The mutated 3' UTR (3' UTR MT) contains a mutated sequence which is not complementary to the ssc-miR-122 seed sequence (GUGA). E Relative fluorescence activity of the luciferase constructs harboring the wildtype or mutant 3' UTR of either NFAT5 or NPEPPS genes in PK15 cells with or without the ssc-miR-122 mimic. SE from the mean are labeled on the bar using vertical lines. *, ** and *** indicate significance at the P value threshold levels of 0.05, 0.01 and 0.001, respectively.
UTR of the two genes (P < 0.001) (Figure 5E). Altogether, these results indicate that both the NFAT5 and NPEPPS genes are regulated by ssc-miR-122 through binding to their 3′ UTR.

Discussion

The susceptibility to PCV2 infection differs among pig breeds. According to our previous study, PCV2-infected YL pigs show serious lesions in lung tissues, while infected LW pigs exhibit no such clinical characteristics. Using RNA-seq, the differentially expressed genes between infected and uninfected LW and YL pigs, including SERPINA1, were identified in the lung tissues [13]. The PCV2 DNA does not express miRNA in subclinically infected tonsils and mediastinal lymph nodes; however, after the PCV2 infection, eight differentially expressed miRNA encoded by the porcine genome were detected in mediastinal lymph nodes [12]. Furthermore, an in vitro PCV2 infection study using the PK15 cells also detected tens of miRNA induced by the expression of PCV2-encoded ORF1, ORF2 and ORF3 proteins [11]. Due to the involvement of miRNA in PCV2 infection, we compared the miRNA expression profiles in the lung tissues of infected and uninfected LW and YL pigs. In the present study, 295 known porcine miRNA were detected in the present lung tissues, and more differentially expressed miRNA were detected in the LW pigs than in the YL pigs after the PCV2 infection. Of these miRNA, miR-132, miR-187 and miR-451 were also detected in the PK15 cells expressing the PCV2-encoded ORF proteins [11]. Moreover, 95 novel miRNA candidates were identified for the first time.

In this study, the expression patterns of five differentially expressed miRNA (i.e., ssc-miR-122, ssc-miR-451, ssc-miR-486, ssc-miR-504 and ssc-miR-192) were validated using sRNA sequencing data. Of these miRNA, ssc-miR-122, ssc-miR-451, ssc-miR-486 and ssc-miR-192 were up-regulated after the PCV2 infection, while the expression level of ssc-miR-504 was down-regulated. By analyzing sRNA libraries constructed from mediastinal lymph nodes of PCV2-infected and uninfected pigs, Nuñez-Hernández et al. detected five up-regulated and three down-regulated miRNA in PCV2-infected pigs [12]. However, none of the eight differentially expressed miRNA were found in our study investigating PCV-infected lung tissues. This difference is likely due to the tissue-specific nature of miRNA expression. However, the miTOR and leukocyte transendothelial migration signaling pathways associated with the four up-regulated miRNA in our study were also reported by Nuñez-Hernández et al. [12], although the miRNA affected by PCV2 were different.

Of the four up-regulated miRNA, miR-122 was further analyzed because its expression is greatly stimulated in LW pigs after PCV2 infection, while it is stable in YL pigs. Porcine miR-122 was first identified to be specifically expressed in the liver tissue [28, 29]. Subsequently, miR-122 was also found to be expressed in the porcine back-fat [30] and longissimus dorsi muscle tissues. In intestinal samples collected from pigs during hypothermic circulatory arrest, the expression of miR-122 was significantly increased [31]. In an ischemic porcine cardiogenic shock model, therapeutic hypothermia significantly reduced the levels of miR-122 [32], and plasma miR-122 was associated with acute coronary syndrome [33]. In this study, miR-122 was expressed in the porcine lung tissue. These data collectively indicate that miR-122 is also expressed in tissues other than the liver.

Regarding the relationship between miR-122 and viral infection, most studies have focused on human hepatitis C virus (HCV). In humans, miR-122 is reported to enhance the accumulation of HCV RNA by binding to the 5′ UTR of the HCV genome [34]. Moreover, the 3′ region of the HCV genome targeted by miR-122 is involved in regulating different steps of the HCV replication cycle [35]. Our results also indicate that ssc-miR-122 could decrease PCV2 viral DNA replication and protein synthesis in PK15 cells. We subsequently analyzed the 1.7 kb genomic sequence of porcine PCV2 virus and did not find binding sites for ssc-miR-122, suggesting that miR-122 cannot directly interfere with the replication of PCV2 virus by targeting genes encoded by the PCV2 virus. We further examined host genes that are likely targets of miR-122 and found that NFAT5 and NPEPPS were negatively regulated by miR-122 through binding to their 3′ UTR regions.

NFAT5 is a member of the NFAT family of transcription factors and regulates gene expression induced by osmotic stress in mammalian cells. NFAT5 was shown to be up-regulated in lung adenocarcinoma cells, and the knockdown of NFAT5 decreased the proliferation and migration of these cells [36]. In this study, after the PCV2 infection, the NFAT5 expression was decreased due to the increased expression of miR-122 in the lung tissue, which is consistent with the lesions observed in the lung tissue after the PCV2 infection [13]. In humans, NFAT5 plays a crucial role in the regulation of HIV-1 replication by directly interacting with a highly conserved long terminal repeat site and the viral promoter [37] and is also modulated by nonstructural 5A for HCV propagation [38]. NPEPPS encodes puromycin-sensitive aminopeptidase, which is a zinc metallopeptidase that hydrolyzes amino acids from the N-terminus of its substrate. In the lung cancer cell line PGCL3, miR-614 inhibited lung...
cancer cell invasion and proliferation by targeting the 3′ UTR of the NPEPPS gene [39]. Further investigations are needed to determine whether NFAT5 and NPEPPS play direct roles in the regulation of PCV2 replication.

In conclusion, the miRNA profile in the porcine lung tissue of PCV2-infected and PCV2-uninfected LW and YL pigs was compared. We identified 21 and seven differentially expressed known miRNA in the lung tissues of LW and YL pigs that were related to the PCV2 infection. The expression levels of ssc-miR-122, ssc-miR-451, ssc-miR-486, and ssc-miR-192 were elevated in the PCV2-infected pigs compared to those in the uninfected pigs, while that of ssc-miR-504 was reduced. In the PK15 cells, ssc-miR-122 can repress the protein expression and viral DNA replication of PCV2, and ssc-miR-122 may down-regulate the expression of NFAT5 and NPEPPS by binding to their 3′ UTR.

Additional files

**Additional file 1.** Percentages of small non-coding RNA mapped to the *Sus scrofa* reference genome. *N* number of clean reads.

**Additional file 2.** Length distribution and abundance of sequences in LW-u (A), LW-i (B), YL-u (C) and YL-i (D) pigs. The most abundant lengths were 22 nt, followed by 23 nt, 21 nt and 24 nt.

**Additional file 3.** Hierarchical clustering of miRNA expression. This analysis clustered miRNA with similar patterns of expression. Each row represents a specific miRNA, and each column represents a pair of samples. That is, the color of each lattice shows the difference in the expression of a given miRNA between a pair of samples. Take LW-u vs LW-i for example; the red indicates that the expression of a given miRNA in LW-u is higher than that of LW-i; the green indicates that the expression of a given miRNA in LW-i is lower than that of LW-u; and the grey indicates that a given miRNA is not expressed in at least one sample.

**Additional file 4.** Information of the novel miRNA in porcine lung tissues. This excel file contains all information of the 95 novel miRNA, including mature miRNA sequence, precursor sequence, precursor location, minimal free energy, normalized expression value, fold-change and P-value. *A* if the normalized expression of a given miRNA was less than 1 in both samples of a sample pair; the normalized expression of both samples were not shown and the miRNA was removed from the differential expression analysis. *b* Sig-label: **fold-change > 1 or < −1, and P value < 0.01.

**Additional file 5.** Secondary structures of the novel miRNA.

**Additional file 6.** DIANA-miRPath predicted KEGG pathways of four up-regulated miRNA. The y-axis indicates the confidence level that a differential expressed miRNA is enriched to a certain KEGG pathway. Confidence levels were measured by −ln(P-value) and correlated positively. Union group indicates the pathway analysis of a combination of all four miRNA.

Abbreviations

PCV2: porcine circovirus type 2; PCV1: porcine circovirus type 1; PRRSV: porcine reproductive and respiratory syndrome virus; PPV: porcine parvovirus; HCV: hepatitis C virus; miRNA: microRNA; sRNA: small RNA; LW pig: Landrace pig; YL pig: Yorkshire x Landrace crossbred pig; LW-u: PCV2-infected LW pig; LW-i: PCV2-uninfected LW pig; YL-u: PCV2-infected YL pig; YL-i: PCV2-uninfected YL pig; NFAT5: nuclear factor of activated T-cells 5; NPEPPS: aminopeptidase puromycin sensitive; 3′UTR: 3′untranslated region; qRT-PCR: quantitative real-time PCR; RNA-seq: RNA-sequencing; MOI: multiplicity of infection; TCID50: 50% tissue culture infective dose; SE: standard error.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YI and YS conceived and designed the experiments. PZ and LW performed the experiments and analyzed the data with assistance and advice from: YL, PJ, YW, PW, LX and YW. PZ, LW and YS drafted the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The data of miRNA-seq generated during the current study are available in NCBI Gene Expression Omnibus (GEO) repository (Accession Number: GSE92259) [40], and other datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Ethics Committee of Shandong Agricultural University and were performed in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, China) (Permit Number: NO. 2007005).

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Author details

1 Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Shandong Agricultural University, 61 Daizong Street, Taian 271018, Shandong, China. 2 College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China.

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