Difference in microRNA expression and editing profile of lung tissues from different pig breeds related to immune responses to HP-PRRSV

Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating diseases for the pig industry. Our goal was to identify microRNAs involved in the host immune response to PRRS. We generated microRNA expression profiles of lung tissues from Tongcheng or Landrace pigs infected with a highly pathogenic PRRS virus (PRRSV) at 3, 5, 7 dpi (day post infection) and control individuals from these two breeds. Our data showed that 278 known and 294 novel microRNAs were expressed in these combined microRNA transcriptomes. Compared with control individuals, almost half of the known microRNAs (116 in Tongcheng and 153 in Landrace) showed significantly differential expression (DEmiRNAs) at least once. The numbers of down-regulated DEmiRNAs were larger than the corresponding number of up-regulated DEmiRNAs in both breeds. Interestingly, miR-2320-5p, which was predicted to bind to conserved sequences of the PRRSV genome, was down-regulated significantly at 3 dpi after PRRSV infection in both breeds. In addition, PRRSV infection induced a significant increase of microRNA editing level in both breeds. Our data showed that 278 known and 294 novel microRNAs were expressed in these combined microRNA transcriptomes. Compared with control individuals, almost half of the known microRNAs (116 in Tongcheng and 153 in Landrace) showed significantly differential expression (DEmiRNAs) at least once. The numbers of down-regulated DEmiRNAs were larger than the corresponding number of up-regulated DEmiRNAs in both breeds. Interestingly, miR-2320-5p, which was predicted to bind to conserved sequences of the PRRSV genome, was down-regulated significantly at 3 dpi after PRRSV infection in both breeds. In addition, PRRSV infection induced a significant increase of microRNA editing level in both breeds. Our results provide novel insight into the role of microRNA in response to PRRSV infection.

MicroRNAs, with a length ranging from 21–24 nucleotides, are important post-transcriptional regulators playing critical roles in cell development and immune response. Recent studies showed that microRNA affected replication of various viruses through binding to the genome of the viruses or regulating host antiviral pathways. The potential role of microRNAs on PRRSV’s replication and host immune pathways upon PRRSV infection has been reported. For example, miR-181 and miR-23a inhibited PRRSV replication through binding to PRRSV genome, while miR-181, miR-125b and miR-506 suppressed PRRSV replication through regulating host antiviral pathways. Porcine alveolar macrophages (PAMs) are the target cells of PRRSV. Using high throughput sequencing technology, recent studies have identified 40 microRNAs involving in host immune response to PRRSV in PAMs in vivo need to be elucidated, e.g., by high throughput sequencing technology.
RNA editing is another important post-transcriptional modification that generates diversity between RNA and genomic DNA sequences. Evidence of RNA editing is evident in the transcripts of humans, rhesus macaques and mice. Adenosine (A) to inosine (I) conversion is the most prevalent type of RNA editing, which is catalyzed by ADAR (Adenosine deaminase acting on RNA) enzymes with double-stranded (ds) RNAs as substrates. Accumulated data supports that microRNA editing plays prominent roles in many biological processes, including virus-hosts interactions. For example, RNA editing is increased due to the elevated expression of ADARs resulting in the Hepatitis Delta Virus replication inhibition. Human cytomegalovirus (HCMV) infection induces the expression of ADAR1-p110, which regulates the editing of miR-376. In human cells, the Epstein-Barr virus (EBV) BART6 miRNA, which targets the Dicer nuclease that cleaves pre-miRNAs into miRNA duplexes, affects the latent state of EBV infection. A-to-I editing of the BART6 miRNA can suppress processing of pre-miRNAs to mature miRNAs by inhibiting Dicer cleavage in the cytoplasm, and thus is potentially an important determinant in the regulation of EBV replication and latency. Thus our interest is in the relationship between microRNA editing and PRRSV replication and host immune response to PRRSVs.

Herein, we identified microRNAs related to PRRSV replication and host immune responses using eight lung microRNA transcriptomes from Tongcheng or Landrace pigs infected with a highly pathogenic PRRSV. We identified the microRNAs that could bind to the PRRSV genome and candidate editing sites on microRNA sequences. Taken together, our results suggest critical and novel roles of microRNAs in the interactions between hosts and PRRSV.

Results
MicroRNA expression profiles of lung tissues after PRRSV infection. We generated eight microRNA expression profiles of lung tissues from Tongcheng or Landrace pigs infected with PRRSV (WU13) at 3, 5 and 7 dpi and control individuals from the two breeds. The numbers of raw reads of these eight transcriptomes ranged from 18 to 30 million (Table 1). After filtering for reads with low quality and removing adaptor sequences contained in the raw reads (see Methods), the number of reads from these eight lung microRNA transcriptomes decreased to 14 to 25 million. Of the total 160 million clean reads, ~80% of them had a length of 21–24 nucleotides (Supplementary Figure S1). We then aligned them to the pig genome (Ensembl: Sscrofa10.2.71) using BWA (Burrows-Wheeler Aligner); approximately 56% to 86% mapped to the pig genome (Table 1). Unique mapped reads were used to annotate small RNAs against databases: Rfam-11.0, rnammer-1.2, snoRNA-LBME, GenomictRNAdatabase and miRBase-release19. Among the annotated small RNAs: rRNA, tRNA, snoRNA, Y_RNA and microRNA, more than 50% of the reads in each library were assigned as microRNA (Supplementary Figure S2). Detailed analyses suggested that 248 to 261 microRNAs were expressed in lung tissues of Tongcheng pigs, 249 to 261 microRNAs were expressed in lung tissues of Landrace pigs, and 239 microRNAs were transcribed in all the eight libraries. Combining results from all eight libraries, we obtained 278 known microRNAs. Moreover, we predicted novel microRNAs using mirmap 2.0 software. Novel microRNA (Supplementary Table S1) prediction showed that 136 to 285 novel microRNAs were expressed in the lung tissues of Tongcheng pigs, 159 to 308 novel microRNAs were expressed in the lung tissues of Landrace pigs, while 294 novel microRNAs were transcribed in the combined eight libraries (Table 1).

Identification of differentially expressed microRNAs after infection. The relative expression levels of microRNAs were normalized as TMM (Trimmed Mean of M-values) using edgeR package. Compared with control groups, we defined microRNAs with a fold change (FC) > 2 and false discovery rate (FDR) < 0.05 as significantly differentially expressed microRNAs (DEmiRNAs). For simplicity, compared with control groups, we referred microRNAs having significantly differential expression in PRRSV-infected Tongcheng pigs at 3, 5 and 7 dpi as set 1, set 2 and set 3, respectively. We merged DEmiRNAs of set 1, set 2 and set 3 into set 4. Similarly, we referred DEmiRNAs in PRRSV-infected Landrace pigs at 3, 5 and 7 dpi compared with control animals of this breed as set 5, set 6 and set 7, respectively. We also merged DEmiRNAs of set 5, set 6 and set 7 into set 8. Detailed analysis on the up-/down-regulated DEmiRNAs showed that the amounts of down-regulated DEmiRNAs were more than the amounts of up-regulated in all the six sets (set 1: 36 (down) vs 16 (up); set 2: 36 (down) vs 26 (up); set 3: 51 (down) vs 25 (up); set 5: 57 (down) vs 15 (up); set 6: 54 (down) vs 21 (up); set 7: 75 (down) vs 34 (up)) (Fig. 1a), which indicated that down-regulation of DEmiRNAs might be a common strategy for pig responses to HP-PRRSV (High Pathogenic-PRRSV) infection. After that, the top 10 most up-/down-regulated microRNAs at 3, 5, 7 dpi were identified compared with control group (Supplementary Table S2). Ssc-miR-183 was the most up-regulated microRNA consistently at 3 dpi (log₂Fold Change (FC) = 4.93), 5 dpi (log₂FC = 5.33) and 7 dpi (log₂FC = 2.68) in Landraces’ lungs. Ssc-miR-215 was the most down-regulated microRNA at 3 dpi (log₂FC = -5.58), 5 dpi (log₂FC = -7.36) and ssc-miR-122 was the most down-regulated microRNA at 7 dpi (log₂FC = -5.71) in Landraces’ lungs. While, in the lungs of Tongcheng pigs, the most up-regulated microRNAs at 3, 5 and 7 dpi were ssc-miR-490-5p (log₂FC = 2.15), ssc-miR-183 (log₂FC = 2.8) and ssc-miR-215 (log₂FC = 6.2), respectively. Correspondingly, the most down-regulated microRNAs were ssc-miR-4332 (log₂FC = -4.19), ssc-miR-374b-3p (log₂FC = -6.07) and ssc-miR-95 (log₂FC = -2.96).

The numbers of DEmiRNAs in set1, set2, set3 and set4 were 52, 62, 76 and 116. Detailed analysis showed that 20 DEmiRNAs were common in all set1, set2 and set3, suggesting that these DEmiRNAs might play roles in persistent PRRSV infection in the lungs of Tongcheng pigs (Fig. 1a). The numbers of DEmiRNAs in set 5, set 6, set 7 and set 8 were 72, 75, 109 and 153, which was more than that in Tongcheng pigs correspondingly. A total of 33 microRNAs (Table 2) has signif-
Differentially expressed microRNAs (DEmiRNAs) analysis. (a) Common DEmiRNAs among different time points and between the two breeds. (b) Heatmap for DEmiRNAs at 3, 5, 7 dpi compared with control groups in the two breeds. The log2 fold changes were used to plot the heatmap.

---

Functionally annotation of the target genes of specific DEmiRNAs.

We predicted target genes of specific DEmiRNAs in Tongcheng (seven DEmiRNAs) and Landrace (20 DEmiRNAs) pigs using the experimental validated microRNA-targets databases: miRecord (version4), mirTarBase 4.5, TarBaseV5.0 and mirTarPri. This resulted in 78 predicted targets for three out of seven Tongcheng specific DEmiRNAs and 1154 targets for nine out of 20 Landrace specific DEmiRNAs. Gene Ontology (GO) analysis showed that the target genes of DEmiRNAs specific in Tongcheng pigs significantly enriched in regulation of transcription, DNA dependent (FDR = 0.018548), T cell differentiation (FDR = 0.027894) (Supplementary Table S3). These results showed that these DEmiRNAs mainly regulate immune related responses and transcriptional activities. In contrast, the target genes of Landrace specific DEmiRNAs were enriched in G protein-coupled receptor signaling pathway (FDR = 9.94e-26) and signal transduction (FDR = 1.89e-06).

---

Significantly differential expression in sets 5, 6, and 7, which was more than that in Tongcheng pigs (20 DEmiRNAs). There were 13 DEmiRNAs were differentially expressed at all the three time points in both pig breeds. Among the 13 DEmiRNAs, only four microRNAs: miR-143-3p, miR-183, miR-219 and miR-28-3p were up-regulated and the other nine microRNAs were all down-regulated in both breeds (Table 2) at all the time points. This result indicated that these microRNAs might be co-regulated during PRRSV infection in both breeds. Next, we merged set 4 and set 8 to obtain 174 DEmiRNAs. The heatmap for these 174 microRNAs' Log2FC revealed that expression patterns of DEmiRNAs in Tongcheng pigs at 3 dpi clustered with those of Landrace pigs at 3 dpi, whereas the DEmiRNAs expression patterns at 5 and 7 dpi were clustered within each breed (Fig. 1b). The dynamic changes of microRNA expression revealed that the two pig breeds had similar microRNA expression pattern at 3 dpi, with different microRNA expression pattern at 5 and 7 dpi. Furthermore, we defined Tongcheng specific DEmiRNAs as the miRNAs differentially expressed significantly at all-time points (3, 5 and 7 dpi) in Tongcheng pigs, but not in Landrace pigs, and vice versa for Landrace specific DEmiRNAs.

Next, we identified seven Tongcheng specific DEmiRNAs and 20 Landrace specific DEmiRNAs. Among the seven Tongcheng specific DEmiRNAs, ssc-miR-204 was the most abundant expressed (Table 3). Specifically, compared with control group, miR-22-5p was significantly up-regulated at 3 dpi (Log2FC = 1.14, FDR = 1.19e-42), 5 dpi (Log2FC = 1.57, FDR = 1.23e-91), and 7 dpi (Log2FC = 1.11, FDR = 1.57e-39) in the lungs of Tongcheng pigs, while not altered significantly in the lungs of Landrace pigs at all the three comparisons. These findings indicated that miR-22-5p might participate in specific responses to PRRSV infection in Tongcheng pigs. Among the 20 specific DEmiRNAs in Landraces' lungs, six DEmiRNAs (ssc-let-7i, ssc-miR-122, ssc-miR-195, ssc-miR-146b, ssc-miR-146a-5p, ssc-miR-30b-5p) had an expression value (TMM) larger than 10,000 at 0 dpi and all of them were down-regulated significantly at 3, 5, 7 dpi (Table 3). These results implied that these six DEmiRNAs might contribute to the Landrace specific responses to PRRSV infection.

We also performed qRT-PCR experiments to validate several DEmiRNAs in mock and infected PAMs (Pulmonary Alveolar Macrophages) from the lungs of Tongcheng and Landrace pig. We examined the microRNA expression level in mock PAMs and infected PAMs with PRRSV WUH3 (MOI = 0.2) at 12, 24, 36 and 48 hpi (hour post infection). The qRT-PCR results showed that the expression patterns of miR-204 and miR-424-5p in the PAMs of Tongcheng pigs after infection with PRRSV WUH3 are similar with those obtained from our deep sequencing data analysis (Fig. 2a, b, e and f). The miR-148a-3p was down-regulated at 3 dpi in our data, but the qRT-PCR results showed that it was up-regulated at all the time point we detected (Fig. 2c, d). High consistency of expression patterns of miRNA-2320-5p and miR-374a-3p were observed between qRT-PCR and the deep sequencing results in Landrace pigs (Fig. 3a, b, c and d). The expression pattern of novel miRNA (5: 5388562-5388582) detected by qRT-PCR was similar with the deep sequencing results though it was up-regulated at 3 dpi in the deep sequencing (Fig. 3e, f). Overall, the six DEmiRNAs we detected all showed similar expression pattern compared with the small RNA sequencing results, which indicated that our analysis results were highly reliable.
KEGG pathway enrichment analysis showed that Tongcheng specific DEmiRNAs’ targets were significantly (p < 0.05) enriched in 24 pathways, while Landrace specific DEmiRNAs’ targets were enriched in 38 pathways (Supplementary Table S4). Among the 38 pathways, cell cycle pathway (p = 5.744e-08) (Fig. 4a) was enriched in Landrace pigs consistent with the GO analysis. However, the Tongcheng specific DEmiRNAs’ targets were not enriched significantly in cell cycle pathway. These findings indicated that cell cycle related pathways were differentially regulated after infection with PRRSV in Landrace pig lungs. Apoptosis is an important defense mechanism to virus infection. 

### Table 2 | Significantly differential expressed microRNAs at all the time points (3, 5, 7 dpi) compared with control individuals in the two breeds

| Time Points | ssc-miR-183 | ssc-miR-219 | ssc-miR-143-3p | ssc-miR-28-3p | ssc-miR-30c-3p |
|-------------|-------------|-------------|----------------|---------------|----------------|
| 0 dpi       | 65          | 1961        | 2588           | 412           | 10670          |
| 3 dpi       | 2610        | 10670       | 6738           | 7063          | 1146757        |
| 5 dpi       | 192555      | 578719      | 1146757        | 974013        |                |
| 7 dpi       | 541         | 1274        | 1301           | 1455          |                |
| 0 dpi       | 40          | 84          | 112            | 131           | 307            |
| 3 dpi       | 639         | 1331        | 244            | 89            |                |
| 5 dpi       | 3863        | 81          | 34             | 1268          |                |
| 7 dpi       | 2335        | 20          | 59             | 58            |                |
| 0 dpi       | 37          | 7           | 9              | 11            |                |
| 3 dpi       | 37          | 7           | 9              | 11            |                |

Note: the “bold” means overlapped DEmiRNAs between the two breeds
sequences from NCBI (Fig. 5a and Supplementary Table S5). The Therefore, we downloaded seven other PRRSV strain genome infection. The targets of Tongcheng, but not Landrace, specific to the control of the viruses to the conserved sequences among different PRRSV strains. play critical roles on their pathogenicity, the microRNA that can bind

As the conserved sequences among different strains of viruses might be important to the control of the viruses’ pathogenicity. The microRNA that can bind

| miRNA               | 0 pi | pi  | 5 pi | pi  |
|---------------------|------|-----|------|-----|
| ssc-miR-204         | 1359 | 588 | 678  | 380 |
| ssc-miR-374b-3p     | 864  | 242 | 13   | 277 |
| ssc-miR-225p        | 406  | 897 | 1206 | 874 |
| ssc-miR-10a-3p      | 186  | 75  | 67   | 59  |
| ssc-miR-345-5p      | 52   | 108 | 191  | 103 |
| ssc-miR-450b-5p     | 49   | 16  | 8    | 13  |
| ssc-miR-491         | 43   | 108 | 103  | 94  |

**Prediction of microRNA binding sites on the conserved regions of PRRSV genome.** Using miRanda v3.3a software, we predicted 243 cellular microRNAs that can bind to WUH3 PRRSV genome (downloaded from http://www.ncbi.nlm.nih.gov/, accession number: HM853673.2). Among them, six microRNAs (ssc-miR-128, ssc-miR-186, ssc-miR-4332, ssc-miR-218-3p, ssc-miR-2320-5p and ssc-miR-150) were predicted to bind to the 3’UTR of WUH3 PRRSV genome. As the conserved sequences among different strains of viruses might play critical roles on their pathogenicity, the microRNA that can bind to the conserved sequences among different PRRSV strains’ genome might be important to the control of the viruses’ pathogenicity. Therefore, we downloaded seven other PRRSV strain genome sequences from NCBI (Fig. 5a and Supplementary Table S5). The sequence conservation can be measured by PhastCons score. We first aligned the eight strains of PRRSV sequences. Then, the PhastCons Score for each base of the genome of WUH3 strain was calculated (Method) (Fig. 5b). The continuous bases (length > 20 bp) with PhastCons Score larger than 0.8 were considered as conserved sequences among the eight strains of PRRSV. If we set the threshold of PhastCons score as 0.7, miR-181 can be predicted to bind to the conserved region, as confirmed previously. We identified 15 conserved regions among the eight PRRSV strains. A total of 14 microRNAs were predicted to bind to these conserved regions of WUH3 PRRSV genome. Among them, miR-2320-5p can bind to 14847 bp–14869 bp on WUH3 PRRSV genome which is close to the 3’UTR (15171 bp–15347 bp) (Supplementary Table S6). Meanwhile, miR-2320-5p also can bind to the 3’UTR (15226 bp–15248 bp) of WUH3 PRRSV genome. In both breeds, miR-2320-5p was down-regulated significantly at 3 dpi (Tongcheng: log2FC = -2.20, p = 7.147e-144, FDR = 3.140e-143; Landrace: log2FC = -2.14, p = 1.132e-269, FDR = 4.758e-269) compared to control. Taken together, we speculated that the expression of miR-2320-5p might be modulated by PRRSV infection due to the potential combination with PRRSV genome.

**Putative microRNA editing during the PRRSV infection.** We identified candidate microRNA editing sites and editing level (Supplementary Table S7) in the lungs from control and infected pigs using previously described methods. A total of 150 and 168 microRNA editing sites (corresponding to 67 and 65 microRNAs) were identified in the lung tissues of Landrace and Tongcheng pigs, respectively (Supplementary Table S8) with 21 microRNAs overlapping. The A-G conversion was the most prevalent among all the eight libraries (Fig. 6a, b). Moreover, we used WebLogo 3.4 to characterize the sequence motif of 10 bp up-/down-stream of the microRNA editing site. In both breeds, we found that at -1 site, G and A were the most prevalent bases and at +10 site, T and A were the most prevalent bases (Fig. 6d). MicroRNA editing level was defined as the ratio of the number of reads supporting the mismatch at the site to the total number of reads detected at this site (Methods). Interestingly, the average RNA editing level in Tongchengs’ lungs gradually increased (T0: 0.14248; T3: 0.14475; T5: 0.15517; T7: 0.16826).
0.28584) during to the time-course of HP-PRRSV infection. In Landrace’s lungs, the average editing level increased from 0 dpi to 5 dpi (L0: 0.21345; L3: 0.26407; L5: 0.28885) and decreased at 7 dpi (L7: 0.24024) compared with 5 dpi, but still higher than 0 dpi (Fig. 6c). Importantly, compared with the controls (0 dpi), the average RNA editing level in microRNAs were significantly increased at 7 dpi (p = 4.88e-07) in Tongcheng’s lungs, and were significantly increased at 3 dpi (p = 0.0061) and 5 dpi (p = 0.00025) in Landraces’ lungs. These findings suggested that infection of WUH3 PRRSV caused a significant increase in average microRNA editing level in lung tissues. Next, we identified 22 and 25 microRNAs that were edited at all the time points in the lungs of Tongcheng pig using qRT-PCR. We randomly chose three DEmiRNAs (miR-204, miR-148a-3p, miR-424-5p) in the lung tissues of Tongcheng pigs from our data. (a), (c) and (e) are the qRT-PCR results; (b), (d) and (f) are TMM values of microRNAs. All the data for qRT-PCR were normalized to U6 and were represented as mean ± SD (n = 3). Statistical significance was analyzed by ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 2 | Validation of DEmiRNAs in PAMs from the lung of Tongcheng pig using qRT-PCR. We randomly chose three DEmiRNAs (miR-204, miR-148a-3p, miR-424-5p) in the lung tissues of Tongcheng pigs from our data. (a), (c) and (e) are the qRT-PCR results; (b), (d) and (f) are TMM values of microRNAs. All the data for qRT-PCR were normalized to U6 and were represented as mean ± SD (n = 3). Statistical significance was analyzed by ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
Tongcheng and Landrace, respectively (Fig. 7a,b). Previous studies implicated that microRNA editing might occur during virus infection. We found almost 20% (13 out of 65) microRNA were edited after infection in lungs of Tongcheng pigs; this was larger than the corresponding ratio 3% (2 out of 67) in lungs of Landrace pigs. Two out of 13 microRNAs (miR-126 and miR-744) were edited in the lungs of Tongcheng at all the three time points (3, 5, 7 dpi) and one microRNA (miR-27a) was edited in the lungs of Landrace at 3, 5, 7 dpi (Fig. 7c). More importantly, miR-181 was also found edited (Supplementary Table S7). Above all, our findings indicated increased microRNA editing levels in PRRSV infected lungs in both pig breeds.

Figure 3 | Validation of DEmiRNAs in PAMs from the lung of Landrace pig using qRT-PCR. We randomly chose three DEmiRNAs (miR-2320-5p, miR-374a-3p, Novel miRNA (5:5388562-5388582)) in the lung tissues of Landrace pigs from our data. (a), (c) and (e) are the qRT-PCR results; (b), (d) and (f) are TMM values of microRNAs. All the data for qRT-PCR were normalized to U6 and were represented as mean ± SD (n = 3). Statistical significance was analyzed by ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
Figure 4 | Target genes of Tongcheng/Landrace specific DEmiRNAs' KEGG pathways enrichment analysis (a) Landrace specific DEmiRNAs target genes enriched in cell cycle pathway. (b) Tongcheng specific DEmiRNA target genes enriched in apoptosis pathway. The red boxes represent the target genes of Tongcheng or Landrace specific DEmiRNAs.
in vivo -0.26), in Landrace pigs possibly due to
Comparison of eight strains of PRRSV using evolutionary
5 0.8) for conservation.
arrest human cells,
scientific reports
were able to enhance the replication of
9 7.44e-
52
REPORTS
5
43x14
SCIENTIFIC
352
combined eight microRNA libraries. Among these,
two breeds. We found 278 known microRNAs expressed in the
PRRSV (3, 5, 7 dpi) and the lung tissues from control groups of these
groups, Tongcheng specific DEmiRNA-22-5p was significantly up-
to PRRSV infection in lung tissues from the two breeds.
Might be co-regulated and play critical roles in immune responses
level of miR-143. These results suggested that these microRNAs
might lead to cell apoptosis through up-regulating the expression
level positively correlated with the expression level of ADAR in dif-
ferent tissues. It is possible that PRRSV infection results in up-
regulation of expression of ADAR leading to elevated RNA editing
after infection. In our study, after infection, the average microRNA
editing levels were higher than control groups in both breeds
(Fig. 7c). Growing evidence suggests that microRNA editing might
involve immune responses to virus infection. During HCMV infec-
tion, edited miR-376 down-regulates the expression of HLA-E which
promotes NK cell ability to eliminate the HCMV infected cells46.
Further study showed that microRNA editing occurred in pre-
miRNA-126, pre-miRNA-744 in Tongcheng and in pre-miRNA-
27a in Landrace at 3, 5 and 7 dpi (Supplementary Table S5). It has
been demonstrated that microRNA editing can dramatically change
target specificity33,35,56 or influence the maturation of microRNAs45.
Although, the editing sites in miR-126, miR-744 and miR-27a were
not in conserved seed sequences, they might affect the processing of
mature microRNAs by down-regulating their expression levels. A
previous study showed that miR-27a can keep inflammation from
over responding47. Compared with control groups, miR-27a was
down-regulated at 3 dpi (log_{2} FC = -0.75), 5 dpi (log_{2} FC = -0.64)
and 7 dpi (log_{2} FC = -0.26), in Landrace pigs possibly due to
microRNA editing altering immune responses to PRRSV infection.
Compared with control group, miR-744 was ~15% down-regulation
in the lungs of Tongcheng pigs at 3 dpi; conversely, it was ~18% up-
regulated in the lungs of Landrace pigs at 3 dpi. Previous studies
showed that TGF-β1 is the direct target of miR-74448. It is possible
that down-regulated miR-744 might up-regulate the expression of
regulator of B-cell homeostasis, TGF-β, which might contribute to
the elimination of PRRSV49.
In summary, comparing the microRNA expression profiles
between Tongcheng and Landrace pigs after PRRSV infection indi-
cated that pathways were differentially regulated between the two pig
breeds. These findings provide insights to the mechanisms about how genetic factors influence the anti-viral strategy through regulating microRNA expression. We first predicted potential microRNA editing events during PRRSV infection; compared with control groups, the significant increase of RNA editing level after infection needs further functional validation. Above all, our results provided novel and crucial roles of microRNAs on PRRSV infection in vivo and provide insights into the mechanisms of PRRSV pathogenicity.

Methods

Ethics statement. All experiments were performed in accordance with relevant guidelines and regulations; the Animal Care and Use Committee of Huazhong Agricultural University approved this research. The samples collection procedures according with the Guidelines of Huazhong Agricultural University, and scientific, ethical and legal principles of the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals which have been accepted world-wide. Animal treatment is in strict accordance with the Recommendations in the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals, 2005, which has been approved by the Department of Science and Technology of Hubei Province in China (Permit Number: SYXX(ER)2010-0029).

Animal challenge, samples collection and small RNA sequencing. A total of 24, 5 weeks old pigs (12 Tongcheng pigs and 12 Landrace pigs), free from PRRSV, were randomly chosen to perform further experiments. Three Tongcheng pigs and three Landrace pigs were randomly selected as control groups. The remaining 18 pigs were inoculated with a high pathogenic PRRSV (WUH3) in muscle at a 3 mL 10^5 TCID50 dose for each pig. At 3, 5, 7 dpi, we randomly slaughtered three Tongcheng pigs and three Landrace pigs. The lung tissues from the slaughtered pigs were collected and were immediately snap-frozen in liquid nitrogen and stored at ~80°C until use. The total RNA from each lung was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's recommendations. RNA quality and quantity were evaluated using Agilent 2100 Bioanalyzer. Equal quantities of RNA isolated from three lungs from three pigs at each time point were pooled. Totally, eight small RNA libraries were constructed according to the standard illumina protocols and were sequenced on solexa platform. The deep sequencing data have been deposited in NCBI SRA database and are accessible through GEO series accession number GSE58436.

The PAMs were obtained by lavaging lungs of 8-weeks-old Landrace and Tongcheng pigs then cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin. Cells were harvested using EASYspin kit produced by Aidlab Biotechnologies Co., Ltd and RNA was extracted following the manufacturer's protocol. U6 RNA was set up as endogenous control. M-MLV Reverse Transcriptase (Takara) was used to reverse transcribes miRNAs. The qRT-PCR was performed in LightCycler® 480 Real-Time PCR System. The primers used for qRT-PCR were listed in Supplementary Table S9.

Alignment and annotation of small RNAs. The raw reads were filtered by following steps: (1) Trimmed the bases with low quality (<20) from 5' end; (2) Discarded the whole reads with average quality less than 20; (3) Clipped adaptor sequence and discarded reads shorter than 18 bp; then, the clean reads were obtained. We used BWA (default set) to align the clean reads on the pig genome (Sscrofa10.2.71). Only the aligned reads mapping uniquely were used for further analysis. Then, all the reads were compared with pig mature microRNA sequences (miRBase release 19) using blastn. The parameters were set to 95% identity and 80% coverage. Blasts and perl scripts were used to calculate the reads count for each microRNA. Novel microRNAs were predicted using miReap 2.0 software. The edget package was used to normalize the expression value of microRNAs (TMM) in different libraries and call differential expressed microRNAs (fold change > 2 and FDR < 0.05). We set the dispersion as 0.1

Figure 6 | The microRNA editing profile during the HP-PRRSV infection in the lungs of Tongcheng and Landrace. (a), (b) Distribution of the number of editing type in the lungs of the two breeds. (c) Average microRNA editing level in control groups (L0, T0) and after infection in the two breeds. The RNA editing level was showed as the ratio of the reads support the mismatch in the site to the total reads detected at this site. The statistic method used in Figure 6c is Kolmogorov-Smirnov test (K-S test). L0 vs. L3: p-value = 0.006148; L0 vs. L5: p-value = 0.000225; L0 vs. L7: p-value = 0.1272; T0 vs. T3: p-value = 0.9997; T0 vs. T5: p-value = 0.07608; T0 vs. T7: p = 4.884e-07.
Target prediction and functional analysis. The targets of microRNAs were extracted from experimental validated miRNA-mRNA databases: miRecord version 4, mirTarBase 4.5, TarBaseV5.0 and mirTarPri. Functional annotation of predicted microRNA targets were performed based on Gene Ontology (GO) database and the pathways that they enriched were analyzed using Kyoto Encyclopedia of Genes and Genomes database (KEGG). The significantly enriched functional categories were determined by the multiple test adjustment proposed by Benjamini & Hochberg. KEGG pathway enrichment analysis and visualization were performed using R packages: GOstats and pathview. The KEGG pathways with a p < 0.01 were defined as significantly enriched pathways.

Prediction of microRNA binding sites on PRRSV genome. We downloaded the genome of WUH3 strain (NCBI accession number: HM853673.2) of PRRSV from NCBI (http://www.ncbi.nlm.nih.gov/). The microRNA binding sites on PRRSV genome were predicted using miRanda v3.3a software. And the coordinates of microRNAs bind sites on PRRSV (WUH3) genome were extracted using perl scripts. We downloaded other seven strains PRRSV's genome sequences from NCBI (Fig. 5a). To identify the conserved regions among the eight strains, the PhastCons scores for each base were calculated (Fig. 5b) by the following steps: first, the muscle 3.8.31 software was used to create alignment fasta documents of PRRSV genomes. And the evolutionary tree was plotted according to the fasta document using FigTree. Second, we used phyloFit to create *mod file, which was the input file for phastCons score calculation; third, phastCons score, was calculated by using phastCons software. Perl scripts were used to extract the consecutive regions with average phastCons score larger than 0.8. We used bedtools intersect function to get the microRNAs that were predicted to bind to the conserved sites on WUH3 genome.

Identification of potential microRNA editing sites. According to the method described in Ref. 37 (detailed in Supplementary), we identified microRNA editing sites located on the known microRNA sequences. The common and specific microRNAs undertook RNA editing across all the time points in the lungs of Tongcheng and Landrace were analyzed using perl scripts. The RNA editing level was showed as the ratio of the reads support the mismatch in the site to the total reads detected on this site. The significance of the pairwise comparisons of average microRNA editing level between control group and infected groups in the two breeds was evaluated using Kolmogorov-Smirnov test. The sequence motif of 10 bp up/downstream around the microRNA editing site in the lungs of Tongcheng and Landrace pigs were analyzed using WebLogo 3.4.

Figure 7 | Common and specific microRNAs undertaken microRNA editing (a),(b) Venn diagram showing the common and specific microRNAs undertook editing in the four time points in the two breeds. (c) Second structure and the editing sites of microRNAs induced by PRRSV infection in the two breeds. The black arrow indicated RNA editing site; The sequence between the purple arrows indicated the mature sequence. (d) Sequence motif for the 10 bp up/downstream of the editing site.

According to previous study61. The false discovery rate was calculated using Benjamini algorithm.

1. Rossow, K. D. Porcine reproductive and respiratory syndrome. *Vet Pathol* **35**, 1–20 (1998).
2. Tian, K. *et al.* Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One* **2**, e526 (2007).
3. Lunney, J. K. & Chen, H. Genetic control of host resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection. *Virus Res* **154**, 161–169 (2010).
4. Petry, D. B. *et al.* Biological responses to porcine respiratory and reproductive syndrome virus in pigs of two genetic populations. *J Anim Sci* **83**, 1494–1502 (2005).
5. Christopher-Hennings, J., Holler, L. D., Benfield, D. A. & Nelson, E. A. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. *J Vet Diagn Invest* **13**, 133–142 (2001).
6. Vincent, A. L. *et al.* An investigation of susceptibility to porcine reproductive and respiratory syndrome virus between two genetically diverse commercial lines of pigs. *J Anim Sci* **84**, 49–57 (2006).
7. Doesch-Wilson, A. B. et al. Clinical and pathological responses of pigs from two genetically diverse commercial lines to porcine reproductive and respiratory syndrome virus infection. J Anim Sci 87, 1638–1647 (2009).
8. Reiner, G., Willems, H., Pesch, S. & Ohlinger, V. F. Variation in resistance to the porcine reproductive and respiratory syndrome virus (PRRSV) in Pietrain and Miniature pigs. J Anim Breed Genet 127, 100–106 (2010).
9. Petry, D. B. et al. Differential immunity in pigs with high and low responses to porcine reproductive and respiratory syndrome virus infection. J Anim Sci 85, 2075–2092 (2007).

10. Boddicker, N. J. et al. A major QTL associated with host response to Porcine Reproductive and Respiratory Syndrome virus challenge. J Anim Sci 90, 1733–1746 (2012).
11. Rowell, B. D., Lunney, J. K. & Dekkers, J. C. M. Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Front Genet 3, 260 (2012).
12. Boddicker, N. J. et al. Validation and further characterization of a major quantitative trait locus associated with host response to experimental infection with Porcine Reproductive and Respiratory Syndrome virus. Anim Genet 45, 182–185 (2014).
13. Boddicker, N. J. et al. Genome-wide association and genomic prediction for host response to Porcine Reproductive and Respiratory Syndrome infection. Genet Sel Evol 46, 18 (2014).
14. Reinhart, B. J. et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403, 901–905 (2000).
15. Chen, C. Z., Schaffner, F., Frangos, B. & Loh, C. Regulation of immune responses and tolerance: the microRNA perspective. ImmunoL Rev 253, 112–128 (2013).
16. Li, Y. & Shi, X. MicroRNAs in the regulation of TLR and RIG-I pathways. Cell Mol Immunol 10, 65–71 (2013).
17. Li, S. et al. MicroRNA-130a inhibits HCV replication by restoring the innate immune response. J Virol Hepat 21, 121–128 (2014).
18. Cheng, J. C. et al. Let-7b is a novel regulator of hepatitis C virus replication. Cell Mol Life Sci 69, 2621–2633 (2012).
19. Trebouag, D. W. et al. RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. Nature 506, 245–248 (2014).
20. Nathans, R. et al. Cellular microRNA and P bodies modulate host-HIV-1 interactions. Mol Cell 34, 696–709 (2009).
21. Guo, X. K. et al. Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. J Virol 87, 1159–1171 (2013).
22. Zhang, Q. et al. MicroRNA-23 inhibits PRRSV replication by directly targeting PRRSV RNA and possibly by upregulating type I interferons. Virology 450–451, 182–185 (2014).
23. Gao, L. et al. MicroRNA 181 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) infection by targeting PRRSV receptor CD163. J Virol 87, 8808–8813 (2013).
24. Wang, D. et al. MiR-125b reduces porcine reproductive and respiratory syndrome virus replication by negatively regulating the NF-kappaB pathway. PLoS One 8, e55838 (2013).
25. Wu, J. et al. MiR-506 inhibits PRRSV replication in MARC-145 cells via CD151. Mol Cell Biochem 389, 275–281 (2014).
26. Hicks, J. A., Yoo, D. & Liu, H. C. Characterization of the microRNAome in porcine reproductive and respiratory syndrome virus infected macrophages. PLoS One 8, e72054 (2013).
27. Bazak, L. et al. A-to-T RNA editing occurs at over a hundred million genomic sites, located in a majority of human genes. Genome Res 24, 365–376 (2014).
28. Li, M. et al. Widespread RNA and DNA sequence differences in the human transcriptome. Science 333, 53–58 (2011).
29. Chen, J. Y. et al. RNA edited in thymus macaque shaped by purifying selection. PLoS Genet 10, e1004274 (2014).
30. Maas, S. et al. Genome-wide evaluation and discovery of vertebrate A-to-I RNA editing. Genome Res 20, 1137–1149 (2010).
31. Nishikura, K. Functions and regulation of RNA editing by ADAR deaminases. Annu Rev Biochem 79, 321–349 (2010).
32. Kawahara, Y. et al. RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. Embo Rep 8, 763–769 (2007).
33. Liu, W. H. et al. ADAR2-mediated editing of mir-214 and mir-122 precursor and antisenic RNA transcripts in liver cancers. PLoS One 8, e81922 (2013).
34. Blows, M. J. et al. RNA editing of human microRNAs. Genome Biol 7, R27 (2006).
35. Alon, S. et al. Systematic identification of edited microRNAs in the human brain. Genome Res 22, 1133–1140 (2012).
36. Samuel, C. E. Adenosine deaminizes acting on RNA (ADARs) are both antiviral and proinflammatory. Virology 411, 180–193 (2011).
37. Jayan, G. C. & Casey, J. L. Increased RNA editing and inhibition of hepatitis delta virus replication by high-level expression of ADAR1 and ADAR2. J Virol 76, 3819–3827 (2002).
38. Nachmani, D. et al. MicroRNA editing facilitates immune elimination of HCMV infected cells. PLoS Pathog 10, e1003963 (2014).
39. Lei, T. et al. Perturbation of biogenesis and targeting of Epstein-Barr virus-encoded miR-BART3 microRNA by adenosine-to-inosine editing. J Gen Virol 94, 2739–2744 (2013).
40. Izaac, H. et al. Editing of Epstein-Barr virus-encoded BART6 microRNAs controls their dicer targeting and consequently affects viral latency. J Biol Chem 285, 33358–33370 (2010).
