

Construction and Analysis for Dysregulated lncRNAs and mRNAs in Lipopolysaccharide-Induced Porcine Peripheral Blood Mononuclear Cells

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Research

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Abstract

Background: Lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria induces an intense inflammatory response in pigs. Long noncoding RNAs (lncRNAs) are emerging as key regulators in inflammation and immunity. However, their functions and profiles in LPS-induced inflammation in pigs are largely unknown. In this study, we profiled global lncRNA and mRNA expression changes in porcine peripheral blood mononuclear cells (PBMCs) treated with lipopolysaccharide (LPS) using lncRNA-seq technique.

Result: Totally 43 differentially expressed (DE) lncRNAs and 1082 DE mRNAs were identified in the porcine PBMCs after LPS stimulation. Functional enrichment analysis on DE mRNAs indicated that these genes were involved in inflammation-related signaling pathways, including cytokine-cytokine receptor interaction, TNF, NF- κ B, Jak-STAT and TLR signaling pathways. Additionally, co-expression network and function analysis identified the potential lncRNAs related to inflammatory response and immune response. The expression of eight lncRNAs (ENSSSCT00000045208, ENSSSCT00000051636, ENSSSCT00000049770, ENSSSCT00000050966, ENSSSCT00000047491, ENSSSCT00000049750, ENSSSCT00000054262 and ENSSSCT00000044651) was validated in the LPS- treated and non-treated PBMCs by quantitative real-time PCR (qRT-PCR). In LPS-challenged piglets, we identified that three lncRNAs (ENSSSCT00000051636, ENSSSCT00000049770, and ENSSSCT00000047491) were significantly up-regulated in liver, spleen and jejunum tissues after LPS challenge, which revealed that these lncRNAs might be important regulators for inflammation .

Conclusion: This study provides the first lncRNA and mRNA transcriptomic landscape of LPS-mediated changes in porcine PBMCs. These findings may provide potential insights into lncRNAs involved in regulating immune and inflammation in pigs.

Background

Gram-negative bacteria causes a serious disease of pigs that is a major threat to swine production industries in china [1]. Endotoxin/lipopolysaccharide (LPS), which comes from the cell wall of gram-negative bacteria, stimulates macrophage/monocyte cells to produce pro-inflammatory cytokine (e.g., TNF- α , IL-1 β and IL-6) [2]. In inflammatory processes, LPS induces Toll-like receptor (TLR) 4 signaling with activation of the TLR4/NF- κ B pathway and the nod-like receptor family protein 3 (NLRP3) inflammasome [3, 4]. However, considering the complexity in the LPS induced inflammatory response, other levels of regulation may also be involved.

Long non-coding RNA (lncRNA) are transcripts longer than 200 nucleotides, which have been implicated in diverse functions, including transcriptional regulation in cis or trans, organization of nuclear domains, and regulation of proteins or RNA molecules [5]. lncRNAs are emerging as key regulators in inflammation. For example, lncRNA-HOTAIR is upregulated in cardiomyocytes of LPS-induced sepsis mice and induces TNF- α production through NF- κ B activation [6]. lncRNA-THRIL is overexpressed in the

LPS-stimulated cells, and aggravates LPS-induced injury possibly via sponging miR-34a [7]. LncRNA-MALAT1 is induced by IL-6 in LPS-treated cardiomyocytes and its overexpression can enhance TNF- α expression via activation of SAA3 [8]. The LPS-induced lncRNA Mirt2 functions as a repressor of inflammation through inhibition of TRAF6 oligomerization and auto-ubiquitination [9]. LncRNA GAS5 reverses LPS-induced inflammatory injury in ATDC5 chondrocytes by inhibiting the NF- κ B and Notch signaling pathways [10]. However, the definition of functional lncRNA in pigs are still limited, partly due to their low sequence conservation and lack of identified shared properties across species [11].

Considering the role of lncRNAs in the inflammatory response, the present study was designed to discover and explore lncRNA expression profile of porcine PBMCs in response to LPS. To date, there has been no systematic attempt to identify the lncRNAs whose expression is changed after the induction of the innate immune response in pigs. This analysis of lncRNA expression changes in the porcine PBMCs after LPS stimulation would contribute to the current knowledge of lncRNA functions in gram-negative bacterial infection disease pathogenesis in pigs.

Methods

Cell isolation, culture and stimulation

The PBMCs from the blood of the Duroc \times Landrace \times Large White (DLW) crossbred piglets (~ 15 kg, ~ 8-week-old) were isolated by Ficoll-Hypaque density gradient centrifugation at room temperature [12]. The PBMCs were cultured in RPMI 1640 medium (Gibco, Australia) supplemented with 10% heat-inactivated FBS (Gibco, Australia), 2 mmol/L L-glutamine, 100 U/mL penicillin and streptomycin (Gibco) at 37 °C under 5% CO₂. LPS (*E. coli* serotype 026:B6, Sigma Chemical, St Louis, MO, USA) was dissolved (10 μ g/mL) in saline solution. The PBMCs were cultured at a concentration of 1×10^7 /mL per well of the 6-well plate, and were treated with LPS (the final concentration was 1 μ g/mL) for 4, 8, 12 and 24 h. Cells were further centrifuged for 10 min at 4000 rpm and harvested for RNA extraction.

Animals and tissues collection

A total of 42 (7.1 \pm 0.9 kg) weaned piglets (Duroc \times Landrace \times Large White) were randomly divided into 7 treatments (6 pigs per treatment). The piglets were injected with 100 μ g/kg body weight (BW) LPS, and slaughtered at 0 h (before LPS challenge), 1, 2, 4, 8, 12 and 24 h (after LPS challenge). The various tissues samples were dissected and snap-frozen in liquid nitrogen.

Quantitative RT-PCR

Total RNA was isolated from cell and tissues samples using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA synthesis and quantitative real-time PCR (qRT-PCR) were carried out as previously described [13]. Expression of mRNA and lncRNA was analyzed using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal normalization control. All data were analyzed using the $2^{-\Delta\Delta CT}$ method [14]. Sequences of specific primers are shown in Additional file 1.

Cytokine TNF- α measurement

The concentration of TNF- α in the supernatants of PBMCs were measured using commercially available porcine ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instruction.

High-throughput sequencing

RNA quality was examined by gel electrophoresis and only paired RNA with high quality was used for lncRNA-seq. lncRNA-seq libraries were prepared according to the manufacturer's instructions and then applied to sequencing on Illumina HiSeq 3000 in Shanghai Genergy Co. Ltd (Shanghai, China). The original reads were harvested from the Illumina HiSeq sequencer. 3' adaptor-trimming and low-quality removal was performed with Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), after which the resulting clean reads were used for lncRNA analysis. Clean reads were aligned to the pig reference genome (Sscrofa11.1) using STAR software (<https://github.com/alexdobin/STAR>).

lncRNA and mRNA differential expression analysis

To determine lncRNA and mRNA differential expression between the LPS-stimulated and unstimulated groups, the expression of each transcript was normalized to the total number of reads in the samples using the following formula: FPKM = total fragments / mapped reads (millions) * exon length (KB). The fold change in lncRNA or mRNA reads was presented as log₂ transformation using the following formula: Fold change = log₂ (LPS/Control). A adjust *P*-value less than 0.05 is considered significant.

Genome distribution of differentially expressed (DE) lncRNAs and mRNAs was illustrated with Circos (<http://circos.ca/>).

Functional enrichment analysis

GO enrichment and KEGG analysis of DE mRNA was performed with DAVID 6.8 database (<https://david.ncifcrf.gov/>). Protein-protein interaction network between DE mRNA were analyzed by Ingenuity Pathway Analysis (IPA).

Target gene prediction and co-expression network construction

The predicted potential target genes whose loci were within a 10-kb window upstream or downstream of the lncRNA was considered *cis*-regulated genes. To determine the *trans*-regulated genes of the DE lncRNAs, the lncRNA and mRNA co-expression analysis were performed using Pearson correlation coefficient (PCC) method. The Pearson correlation coefficient was ≥ 0.9 . The common genes between the potential targets of DE lncRNAs and DE mRNAs was analyzed using Venn analysis.

The network of coding-noncoding coexpressed genes was constructed with the biological functions to recognize the novel and significant lncRNA. Correlations between lncRNAs and their corresponding

mRNAs were calculated with Pearson's correlation ($|\text{correlation}| \geq 0.9$) were used to draw the co-expression network through Cytoscape v 3.7.1.

Statistical analyses

The data are shown as means \pm S.D. Differences were tested using ANOVA and the Student's paired t test. The level of significance was set at $P < 0.05$ for all data analysis.

Results

The expression of TNF- α , IL-1 β and IL-6 in LPS induced PBMCs

In order to identify principal LPS-responsive lncRNAs, PBMCs isolated from the whole blood of the three healthy pigs were stimulated with LPS for 4, 8, 12 and 24 h to induce inflammatory response. The mRNA of TNF- α increased significantly in porcine PMBCs within 12 h and peaked at 4 h after LPS stimulation (Fig. 1a). IL-6 and IL-1 β were significantly increased at each time point and peaked at 8 h and 4 h, respectively (Fig. 1b-c). We also applied ELISA to determine TNF- α protein level in the cell culture supernatant. The concentration of TNF- α was obviously increased by LPS stimulation compared to the control at each time point (Fig. 1d), and it was undetected in controls at 12 and 24 h. These results suggested an acute inflammation was induced by LPS stimulation in PBMCs. Then, the RNA samples isolated from PBMCs treated for 8 hours were used for further high-throughput sequencing.

Characters of lncRNA-seq

After quality control, a total of 92550258, 93643366, 107683222, 125384254, 121886474 and 223904698 clean reads with greater than 94.36% of Q30 were obtained in L1, L2, L3, N1, N2 and N3 libraries (Table 1). Among them, a total of 97.60% (L1), 98.20% (L2), 97.90% (L3), 97.30% (N1), 97.50% (N2) and 96.99% (N3) reads from the six libraries were mapped to the pig reference genome (*Sus scrofa* 11.1).

Table 1
Basic data of sequencing in LPS- stimulated and unstimulated PBMCs

Terms	L1 library	L2 library	L3 library	N1 library	N2 library	N3 library
Raw reads	93,474,028	94,531,006	108,795,010	126,394,540	123,017,688	225,897,868
Clean reads	92,550,258	93,643,366	107,683,222	125,384,254	121,886,474	223,904,698
Clean reads rate, %	99.01	99.06	98.98	99.2	99.08	99.12
Clean Q30 bases rate, %	95.11	95.16	95.16	94.36	94.41	94.36
Mapped reads	89,943,602	91,841,971	105,013,431	121,730,896	118,421,869	215,902,508
Mapped rate, %	97.60	98.20	97.90	97.30	97.50	96.99
L1, L2 and L3 represent three experimental libraries (LPS-stimulated PBMCs); N1, N2 and N3 represent three control libraries (unstimulated PBMCs).						

LncRNA and mRNA profile changes in response to LPS stimulation

The whole expression feature of transcripts was shown in Fig. 2a. The expression level of the transcripts in the control was slightly higher than that in LPS-treated PBMCs. A total of 27430 mRNAs and 1074 lncRNAs were obtained from our six libraries (Additional file 2 and 3). Of the 43 DE lncRNAs, 31 were significantly up-regulated and 12 were significantly down-regulated in LPS-stimulated PBMCs compared with the control (Fig. 2b-c,e and Additional file 4). Of the 1082 DE mRNAs, 636 were significantly up-regulated and 446 were significantly down-regulated in PBMCs after LPS stimulation (Fig. 2b, d and Additional file 5).

We next predicted the *cis*- and *trans*-target mRNA of the DE lncRNAs (Additional file 6 and 7) and compared these predictions with our mRNA sequencing results. As shown in Fig. 2f, 621 target mRNAs of up-regulated lncRNAs showed up-regulated expression, while 387 target mRNAs of down-regulated lncRNAs showed down-regulated expression during LPS stimulation.

Basic characteristics of lncRNAs in the PBMCs

The basic characteristics of all DE lncRNAs and DE mRNAs in PBMCs, which are widely distributed in all chromosomes except Y chromosome, are shown in the Circos plot (Fig. 3A). Next, we classified the PBMC lncRNAs into five categories according to the genomic loci of their neighbouring genes (Fig. 3B). Although

10% of lncRNAs were not successfully categorized, the well-annotated lncRNAs were classified into the following categories: intergenic (22%), antisense (9%), intronic (57%) and bidirectional (2%), and sense (0%).

Function analysis of DE mRNAs

The functional enrichment analysis of the 636 significantly up-regulated genes was performed. Our GO analysis included three parts: biological process (BP), cellular component (CC) and molecular function (MF). The top 15 GO enrichment for biological process for the up-regulated mRNAs was illustrated in Fig. 4a, including inflammatory response, immune response, positive regulation of inflammatory response and chemokine-mediated signaling pathway. The significantly enriched GO terms for cellular component and molecular function were identified, such as extracellular space, external side of plasma membrane, integral component of plasma membrane, cytoplasm, I- κ B/NF- κ B complex, cytokine activity, chemokine activity and growth factor activity.

The 446 significantly down-regulated genes were also selected to carry out the functional enrichment analysis. Twenty four GO terms, including protein localization to plasma membrane, regulation of G-protein coupled receptor protein signaling pathway, cell surface, cell-cell junction, and kinase activity, were shown in Fig. 4b.

Meanwhile, KEGG results from the significantly up- and down-regulated genes indicated that the top 30 significantly signaling pathways were enriched (Fig. 4c), such as cytokine-cytokine receptor interaction, TNF signaling pathway, NF- κ B signaling pathway, Jak-STAT signaling pathway, Chemokine signaling pathway and Toll-like receptor signaling pathway.

In addition, the interaction network between proteins was produced using IPA. The inflammatory immune network was shown in Fig. 4d. Seven candidate genes (*NTRK1*, *S100A8*, *S100A9*, *TNIP1*, *TNFAIP3*, *TAX1BP1* and *NOD2*) were screened out as Hub genes.

Function analysis of DE lncRNA and the lncRNA-mRNA co-expression network

A total of 1053 potential target genes of DE lncRNAs were selected to carry out functional enrichment analysis. Our results showed that the top 20 GO enrichment for biological process focused on immune response, inflammatory response, neutrophil chemotaxis, positive regulation of inflammatory response, chemokine-mediated signaling pathway, positive regulation of ERK1 and ERK2 cascade, lymphocyte chemotaxis, positive regulation of NF- κ B import into nucleus, positive regulation of IL-6 production, necroptotic signaling pathway, monocyte chemotaxis, cell chemotaxis, positive regulation of NF- κ B transcription factor activity, negative regulation of IL-10 production and lipopolysaccharide-mediated signaling pathway, etc. (Fig. 5a). Meanwhile, the KEGG results from these target genes of DE lncRNAs were mainly involved in immune response (Fig. 5b).

To identify the key lncRNAs related to the regulation of inflammatory response and immune response, 54 DE mRNAs associated with these two biological processes and 31 DE lncRNAs targeting them were

chosen to build the mRNA-lncRNA co-expression network. The co-expression network comprised 1241 connections and each lncRNA might correlated with multiple mRNAs (Fig. 5c and Additional file 8). More importantly, a total of 29 lncRNAs were found to be co-expressed with chemokines (CCL2, CCL3L1, CCL11, CCL17, CCL20, CCL22, CXCL2, CXCL8, CXCL10 and CXCL13) and cytokines (IL-1A, IL-6, IL-7, IL-10, IL-12B, IL-13, IL-18, IL-19, IL-20, IL23A, IL-27). While, a total of 30 lncRNAs were shown to be co-expressed with other inflammatory-related genes such as Toll-like receptors (TLR2 and TLR3), the Rel/NF- κ B family members (REL, RELB and NFKB2), and NFKBIZ encoding the NF- κ B inhibitor.

qRT-PCR validation of DE lncRNAs in PBMC

We focused on the known lncRNAs and successfully designed 9 DE lncRNA primer pairs for qRT-PCR validation. We identified the 9 DE lncRNAs expressions in porcine PBMCs at 4, 8, 12 and 24 h after LPS treatment. The results were shown in Fig. 6. Four lncRNAs were significantly up-regulated by LPS treatment at all time points, including lncRNA ENSSSCT00000045208, lncRNA ENSSSCT00000051636, lncRNA ENSSSCT00000049770 and lncRNA ENSSSCT00000050966. The expression of lncRNA ENSSSCT00000047491 was significantly up-regulated by LPS treatment at 4, 8 and 12 h, and lncRNA ENSSSCT00000049750 expression was significantly increased at 4 and 8 h. After 8 h of LPS stimulation, the expression of lncRNA ENSSSCT00000054262 was significantly up-regulated and reached the peak at 24 h. lncRNA ENSSSCT00000044651 was significantly increased only at 4 h after LPS stimulation. However, there was no change in lncRNA ENSSSCT00000059583 expression following LPS treatment.

Three lncRNAs (ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636) expressions were increased more than 2.5-fold in LPS-treated PBMCs at 4 h. Therefore, we further identified the expression changes of the three lncRNAs in various tissues of the piglets challenged with LPS at different time points.

Expression of lncRNAs (ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636) in various tissues of piglets challenge with LPS

As shown in Fig. 7a, in the liver tissue, lncRNA ENSSSCT00000047491 were dramatically up-regulated at least 4-fold by LPS from 2 h to 24 h, while the upregulation (> 9-fold) of lncRNA ENSSSCT00000051636 was observed after LPS challenge for 8 hours. lncRNA ENSSSCT00000049770 was dramatically (4 to 12-fold) up-regulated within 8 h but it was down-regulated at 24 h.

As shown in Fig. 7b, in the jejunum tissue, lncRNA ENSSSCT00000049770 was increased significantly at least 1.9-fold from 1 h until 24 h after LPS challenge, while the expression of lncRNA ENSSSCT00000051636 was significantly increased by > 2-fold from 1 h to 4 h. The expression of lncRNA ENSSSCT00000047491 was significantly up-regulated by LPS challenge at 4, 12 and 24 h.

As shown in Fig. 7c, in the spleen tissue, the expression of the three lncRNAs was significantly increased within 8 h and reached the peak at 2 h, but their expression was decreased at 24 h.

As shown in Fig. 7d, in the thymus tissue, the expression of the three lncRNAs showed no significant difference.

Tissue expression pattern analysis of lncRNA ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636

We further detected the differences of the three lncRNAs (ENSSSCT00000049770, ENSSSCT00000051636 and ENSSSCT00000047491) expression levels in piglets varieties tissues by qRT-PCR. The results showed that the three lncRNAs were expressed in all of the ten tissues: skeletal muscle, heart, liver, spleen, lung, kidney, jejunum, stomach, brain and thymus. We also found that the expression levels of lncRNA ENSSSCT00000047491 and ENSSSCT00000051636 were higher in liver and spleen than in other tissues, while the expression level of lncRNA ENSSSCT00000049770 were higher in spleen, lung and jejunum than in other tissues (Fig. 8).

The potential target genes of lncRNAs ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636

We predicted the *cis*-target mRNAs of the lncRNAs ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636 and compared these predictions with co-expression analysis. As shown in Fig. 9, the potential target genes of those lncRNAs included *BIRC5*, *TK1*, *PGS1*, *TMEM235*, *AFMID*, *SYNGR2*, *KREMEN2*, *PRSS33*, *PKMYT1*, *FLYWCH1*, *HCFC1R1*, *TNFRSF12A*, *PAQR4*, *CLDN6*, *THOC6*, *SRRM2*, *PRSS21*, *FLYWCH2*, *CLDN9*, *BICDL2*, *PRSS41* and *ELOB*. However, only *FLYWCH2* was co-expressed with lncRNA ENSSSCT00000049770.

Discussion

Accumulating evidence has indicated that lncRNAs play roles in immune/inflammatory processes [15, 16]. The molecular mechanisms of associated lncRNA responsible for the LPS-induced inflammation in PBMCs remain largely undefined. In human PBMCs, Zhang *et al.* applied a microarray platform to profile global lncRNA and mRNA expression changes in response to LPS, and identified 846 DE lncRNAs (596 up-regulated and 250 down-regulated) and 1,351 DE mRNAs (802 up-regulated and 549 down-regulated), respectively [17]. However, to date there has been no systematic attempt to identify LPS-associated lncRNAs in porcine PBMCs.

In this study, we provides the first lncRNA and mRNA transcriptomic landscape of LPS-mediated changes in porcine PBMCs. Using a sequencing approach, we obtained 1074 lncRNAs and 27430 mRNAs in porcine PBMCs. Of the 1074 lncRNAs, we identified 31 up-regulated and 12 down-regulated lncRNAs in LPS-treated PBMCs compared with the control. Of the 27430 mRNAs, 636 mRNAs were up-regulated and 446 mRNAs were down-regulated by LPS stimulation. Similarly to the results described by Zhang *et al.* [17] in human PBMCs, we found more mRNAs than lncRNAs were dysregulated in response to LPS. Moreover, more transcripts of the dysregulated mRNA or lncRNAs were observed to be up-regulated after LPS stimulation in human and porcine PBMCs. In our recent study, we had investigated the changes in

miRNA expression in porcine PBMCs after in vitro stimulation with LPS and identified only 15 DE miRNA in response to LPS by using small RNA sequencing [12]. Therefore, we thought LPS stimulation might have more influence on protein coding RNA more than non-coding RNA in PBMCs.

It was well recognized that a series of genes involved in LPS-induced inflammation [18–20]. In this study, the DE mRNAs functional enrichment results showed that these genes were related to some biological processes, including inflammatory response, immune response, cytokine activity, chemokine activity, cytokine-cytokine receptor interaction, TNF signal pathway, NF- κ B signal pathway, JAK-STAT signal pathway, NOD-like receptor signal pathway and TLR signal pathway, which were closely associated with LPS-induced inflammation [21–25]. In addition, though the IPA network analysis, we identified some genes might play key roles in LPS-induced inflammation, including NTRK1, S100A8, S100A9, TNFAIP3, TNIP1, TAX1BP1 and NOD2. As a high-affinity receptor for nerve growth factor (NGF), NTRK1 is expressed on various structural and hematopoietic cells including basophils and eosinophils [26, 27]. Rochman *et al.* [28] demonstrated IL-13 confers epithelial cell responsiveness to NGF by regulating NTRK1 levels by a transcriptional and epigenetic mechanism and this process likely contributes to allergic inflammation. Calcium-binding proteins S100A8 and S100A9 have been identified as important DAMPs and recognized by TLR4 on monocytes, which function as innate amplifier of infection, autoimmunity, and cancer [29, 30]. The TNFAIP3 gene, encoding the ubiquitin-modifying enzyme A20, that restricts NF- κ B-dependent signaling and prevents inflammation via its deubiquitinase activity [31]. TNIP1 is increasingly being recognized as a key anti-inflammatory protein by negatively regulating TANK-binding kinase 1 (TBK1), receptor-interacting serine/threonine kinase 1 (RIP1 or RIPK1), and interleukin-1 receptor-associated kinase 1 (IRAK1) [32–35]. TAX1BP1 is a negative regulator of NF- κ B activation induced by TNF- α and IL-1 β [36]. It inhibits RIP1 and TRAF6 polyubiquitination and recruits A20 to these molecules in order to influence NF- κ B activation [37]. NOD2 is a macrophage-specific protein containing two CARD domains and can directly bind bacterial lipopolysaccharide and subsequently act as an activator of NF- κ B via the association of the CARD domains with Rip2/RICK/CARDIAK [38].

LncRNAs can be categorized into five broad subcategories: antisense, sense, intergenic, intronic, and bidirectional [39]. Approximately half of lncRNAs in porcine PBMCs belonged to the intronic subcategory, which describes lncRNAs that are located within protein-coding genes and could regulate functional gene expression. Based on their mode of action on gene expression, lncRNAs can be classified as either cis- or trans-acting. Cis-acting lncRNAs affect the expression of genes located near their site of transcription on the same chromosome. Trans-acting lncRNAs can control gene expression at independent loci on other chromosomes [40]. Then, we predicted the cis and trans potential targets of DE lncRNAs and compared these with our mRNAs sequencing results. The DE lncRNA-associated DE mRNAs were further analyzed for GO category and KEGG pathway annotation to investigate the potential regulatory roles of LPS-mediated DE lncRNAs. Bio-informatics analysis of DE lncRNAs target genes showed that these genes played important roles in immune response, inflammation, positive regulation of ERK1 and ERK2 cascade, positive regulation of NF- κ B import into nucleus, positive regulation of IL-6 production and immune cell chemotaxis. ERK1 and ERK2 are reported to be required for LPS-induced production of cytokines and chemokines by macrophages [41]. KEGG analysis also indicated that the DE lncRNAs were

predominantly associated with the regulation of multiple inflammatory associated genes. In addition, the lncRNA-mRNA co-expression analysis showed revealed that 29 DE lncRNAs targeted CCL and CXCL chemokines, indicating that these lncRNAs might participate in immune cell chemotaxis. The co-expressed network showed that multiple lncRNAs could interact with NFKBIA, which conjugated with NF- κ B resulting in its cytoplasmic sequestration and inhibition of transcriptional activation [42]. Consequently, our results provide new evidence that lncRNAs are involved in LPS-induced inflammation in porcine PBMCs.

Subsequently, 9 DE lncRNAs were selected for validation through qRT-PCR. For the nine lncRNAs, 8 selected lncRNAs were validated to be significantly up-regulated by LPS stimulation at different times. The three lncRNAs (ENSSSCT0000004749, ENSSSCT00000049770 and ENSSSCT00000051636) was increased more than 2.5-fold after LPS stimulation in porcine PBMCs. Then, we further confirmed the expression changes of the three lncRNAs in liver, spleen, jejunum and thymus of the piglets challenged with LPS at different times (0, 1, 2, 4, 8, 12 and 24 h). The previous studies demonstrated LPS challenge can induce severe inflammation in the piglet model, causing liver injury, intestinal damage and histological changes of spleen [43–47]. Although the three lncRNAs displayed different tissue expression patterns in the piglets, they all shown abundant expression in liver, spleen, jejunum and thymus. In liver tissues, lncRNA ENSSSCT00000047491 expression increased approximately 4- to 33-fold, lncRNA ENSSSCT00000049770 increased 4- to 12-fold and lncRNA ENSSSCT00000051636 increased 9- to 64-fold in response to LPS challenge. In the spleen and jejunum, LPS challenge induced an increase in these lncRNAs expression with a maximum response of 4-fold increase. However, in the thymus, LPS challenge had no effect on the expressions of the three lncRNAs. These results indicated the three lncRNAs might have different function in different tissues. We predicted only FLYWCH2 gene was the *cis*-target of lncRNA ENSSSCT00000049770 and also co-expressed with this lncRNA. However, there is very little specific information on the expression and function of FLYWCH2 gene in inflammation.

Conclusions

In the current study, we have provided the changes of lncRNA expression in porcine PBMCs after LPS stimulation, which provided a novel foundation for improving our understanding of the association between PBMC lncRNA homeostasis and inflammatory response in pigs. Further investigations are still required to evaluate the biological functions of these identified lncRNAs and these signaling pathways with regard to their roles in immunity and disease.

Abbreviations

PBMC: Peripheral blood mononuclear cell; DE: Differentially expressed; lncRNAs: Long noncoding RNAs; qRT-PCR: Quantitative real time PCR; TLR: Toll-like receptor; NLRP3: nod-like receptor family protein 3; BW: body weight; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PCC: Pearson correlation coefficient; BP: Biological process; CC: Cellular component; MF: Molecular function (MF); NGF: nerve growth factor; TBK1: TANK-binding kinase 1; IRAK1: interleukin-1 receptor-associated kinase 1

Declarations

Ethics approval and consent to participate

All animal procedures were conducted according to the guidelines for experimental animals established by the HuBei Province, China Biological Studies Animal Care and Use Committee and approved by Wuhan Polytechnic University (Wuhan, China).

Consent for publication

Not applicable.

Availability of data and material

All the data are available in the Sequence Read Archive (SRA) database under the accession number RPJNA656175.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

JZ and YLL conceived and designed the experiment. XX, KP and HLZ performed the experiments. JZ, HBC, and HYR analyzed the data. XX and YLL participated in the collection of samples. JZ wrote the manuscript. All authors read and approved the final manuscript.

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Figures

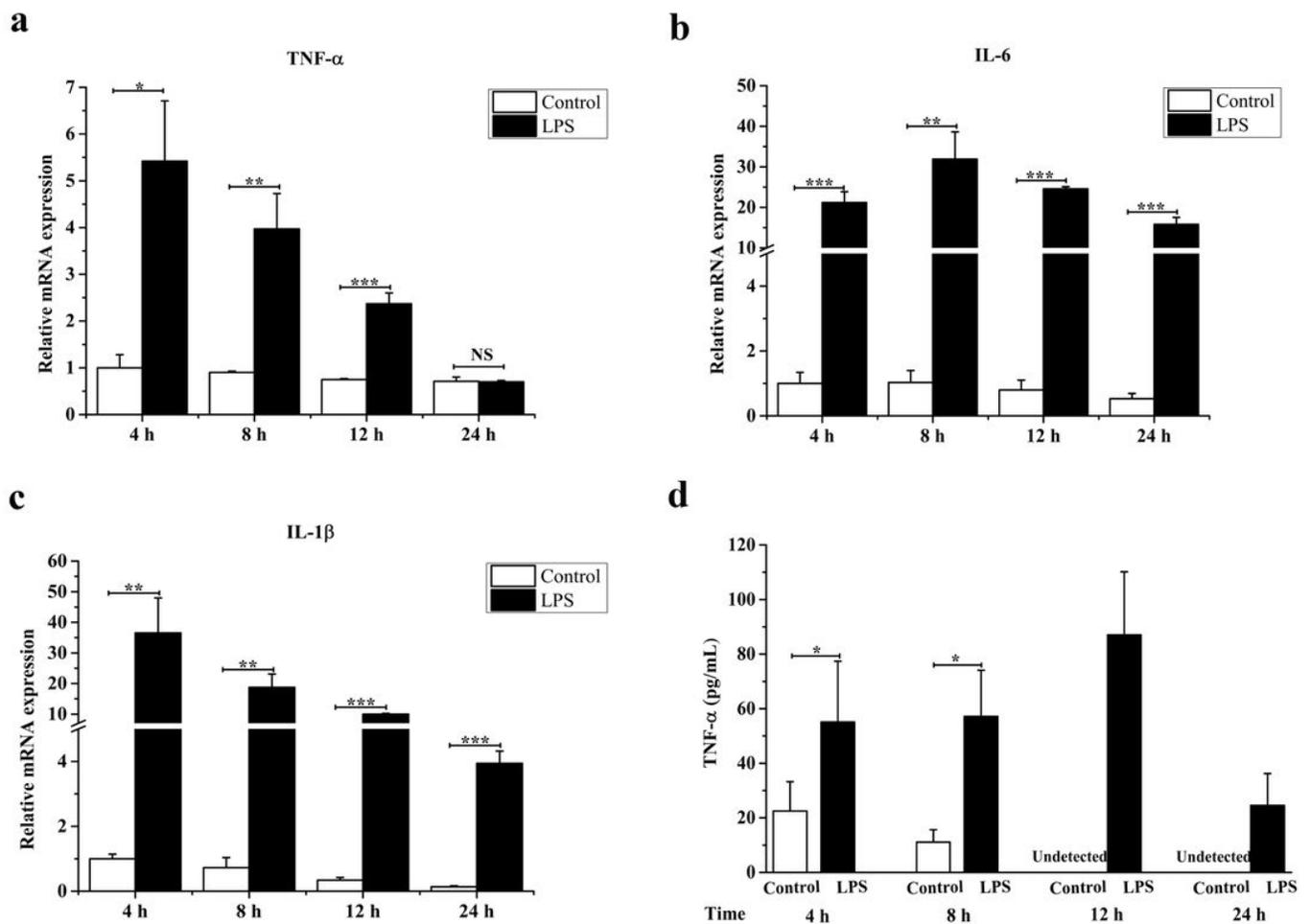


Figure 1

LPS-induced acute inflammation in porcine PBMCs. (a-c) TNF- α , IL-6 and IL-1 β mRNA expression were determined by qRT-PCR in porcine PBMCs at 4, 8, 12 and 24 h after treatment with or without LPS. (d) The concentration of TNF- α in supernatants of the PBMCs were measured at 4, 8, 12 and 24 h after treatment with or without LPS. The data represent the mean \pm SD. N=3. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.

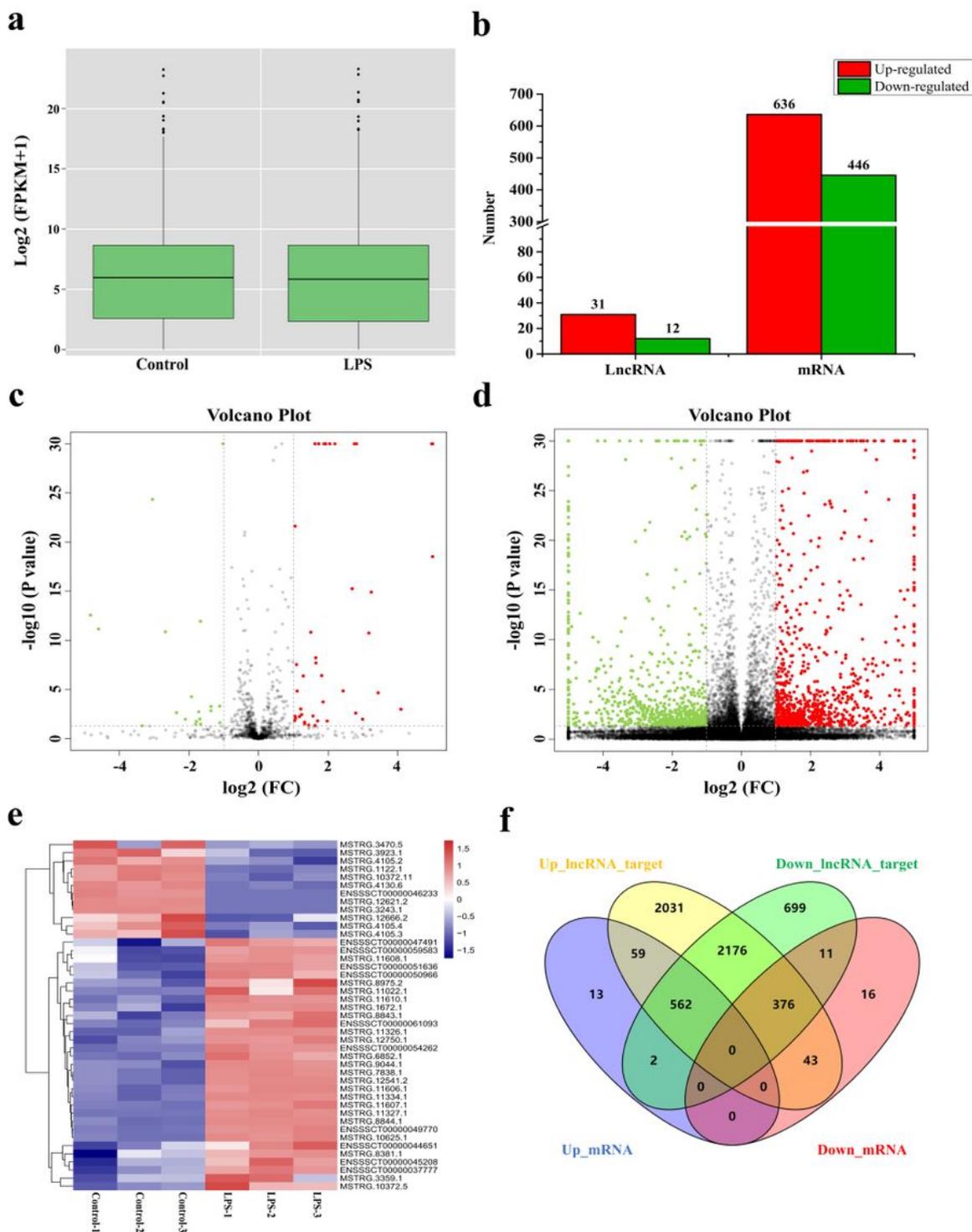


Figure 2

The expression profiling changes of mRNA and lncRNA in LPS-stimulated and -unstimulated PBMCs. (a) The relative expression level of the transcripts in the control and LPS groups. (b) Histogram showing the number of up- and down-regulated lncRNAs and mRNAs in the LPS group compared to the control. (c) Volcano plot indicating up- and down-regulated lncRNAs in LPS group when compared to the control. Red represents up-regulation and green represent down-regulation. (d) Volcano plot indicating up- and down-

regulated mRNA in LPS group when compared to the control. (e) Heat map of lncRNA showing hierarchical clustering of altered lncRNAs in six groups. (f) Venn diagram showing the overlapping number of targeted mRNAs in up-regulated lncRNAs, targeted mRNA in down-regulated lncRNAs, up-regulated mRNAs, and down-regulated mRNAs.

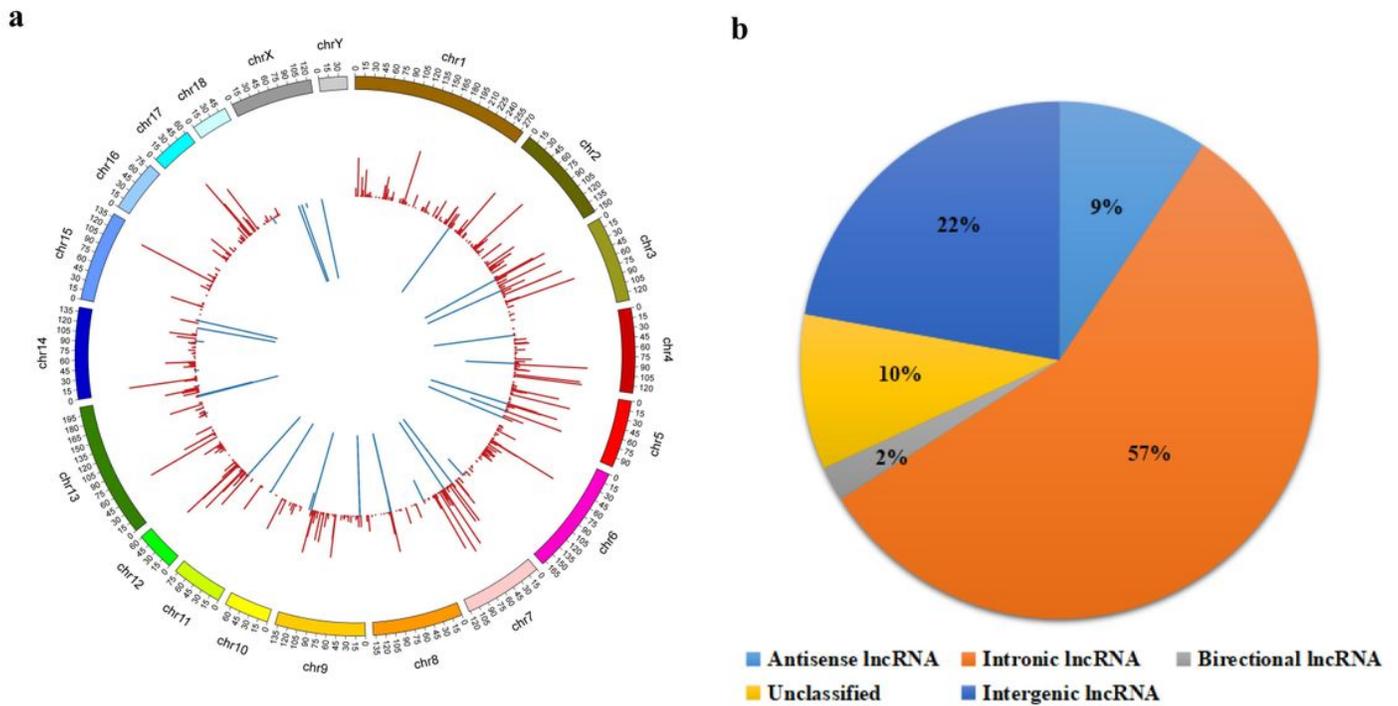


Figure 3

Gene expression characterization. (a) Chromosome distribution of DE lncRNAs and mRNAs by using Circos. Red columns represent DE mRNAs, and blue columns represent DE lncRNAs. (b) The classification annotation of the lncRNAs.

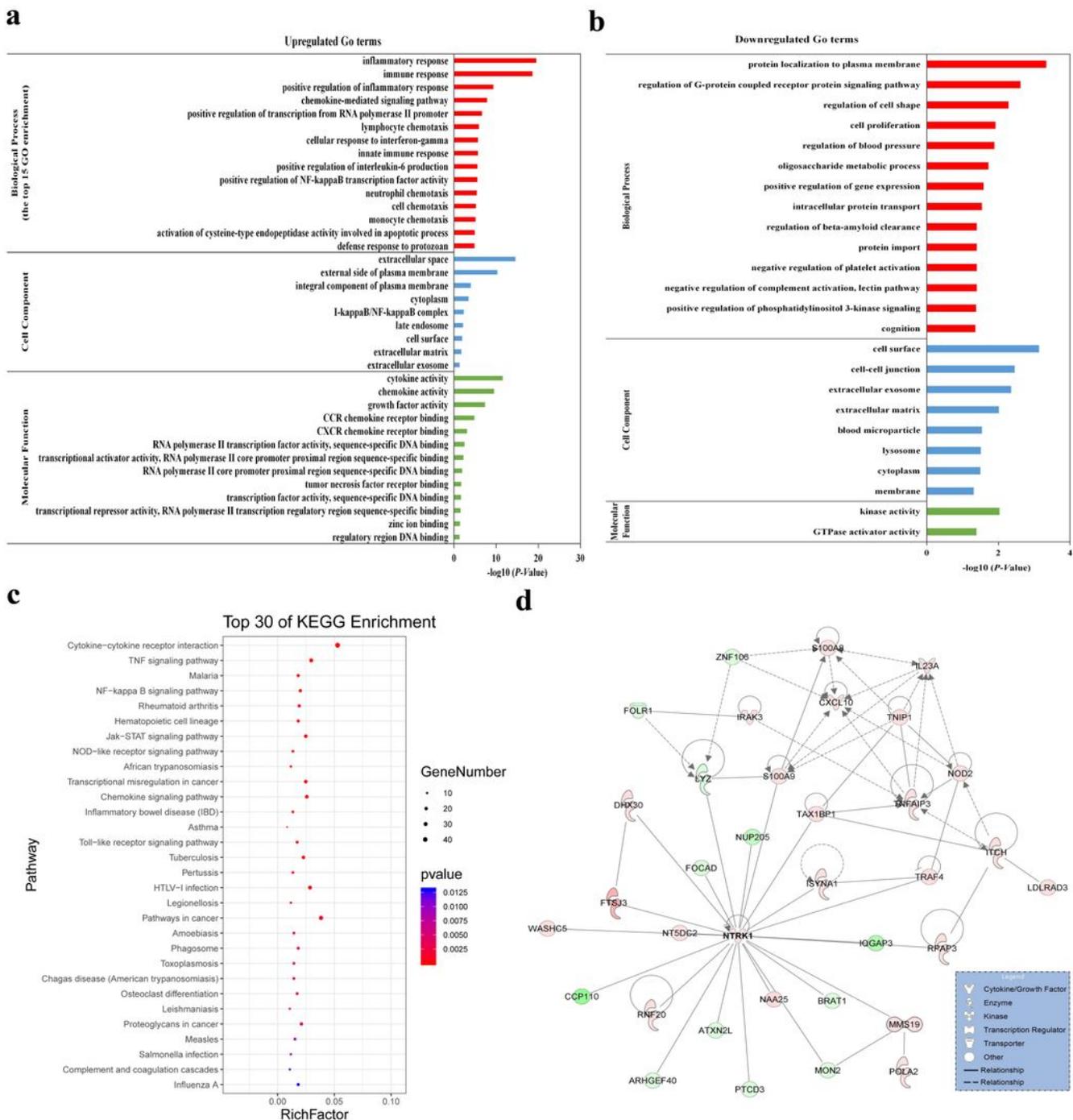


Figure 4

GO term, KEGG pathway and proteins network analysis of DE mRNAs. (a) GO analysis of up-regulated mRNAs. (b) GO analysis of down-regulated mRNAs. The GO terms ranked by P-Value. (c) KEGG pathway enrichment analysis of DE mRNAs. The top 30 enriched KEGG pathways ranked by P-Value. (d) The inflammatory immune network between proteins was produced using IPA.

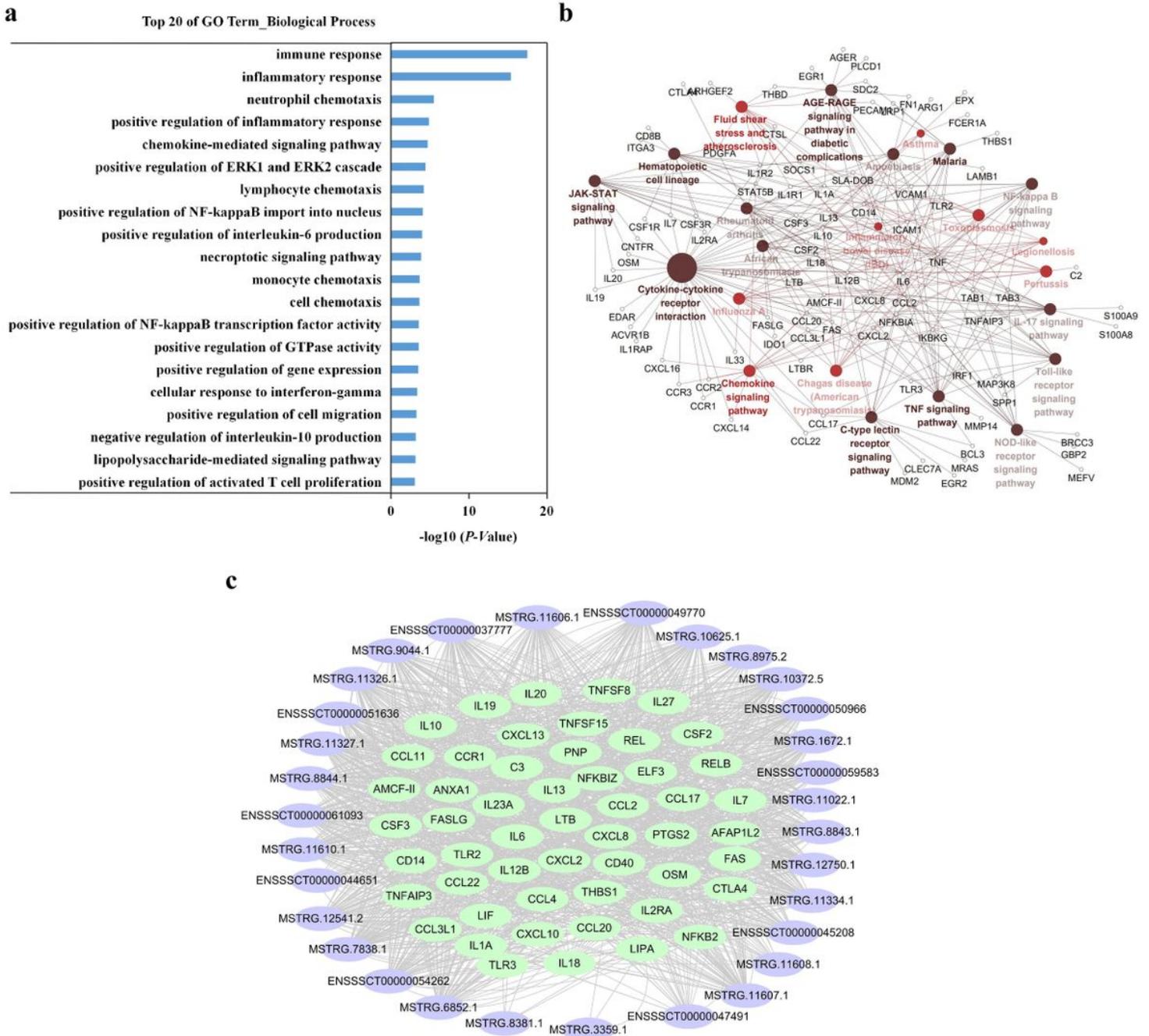


Figure 5

GO term and KEGG pathway analysis of DE lncRNA and the lncRNA-mRNA co-expression network. (a) Biological Process GO term analysis of the potential targets of DE lncRNAs. The top 20 enriched GO terms ranked by P-Value. (b) The KEGG pathway enrichment analysis of the potential targets of DE lncRNA. We display the enriched pathway terms with a P value of < 0.01. (c) The interaction network between candidate lncRNAs and their potential target genes related to inflammation and immune response.

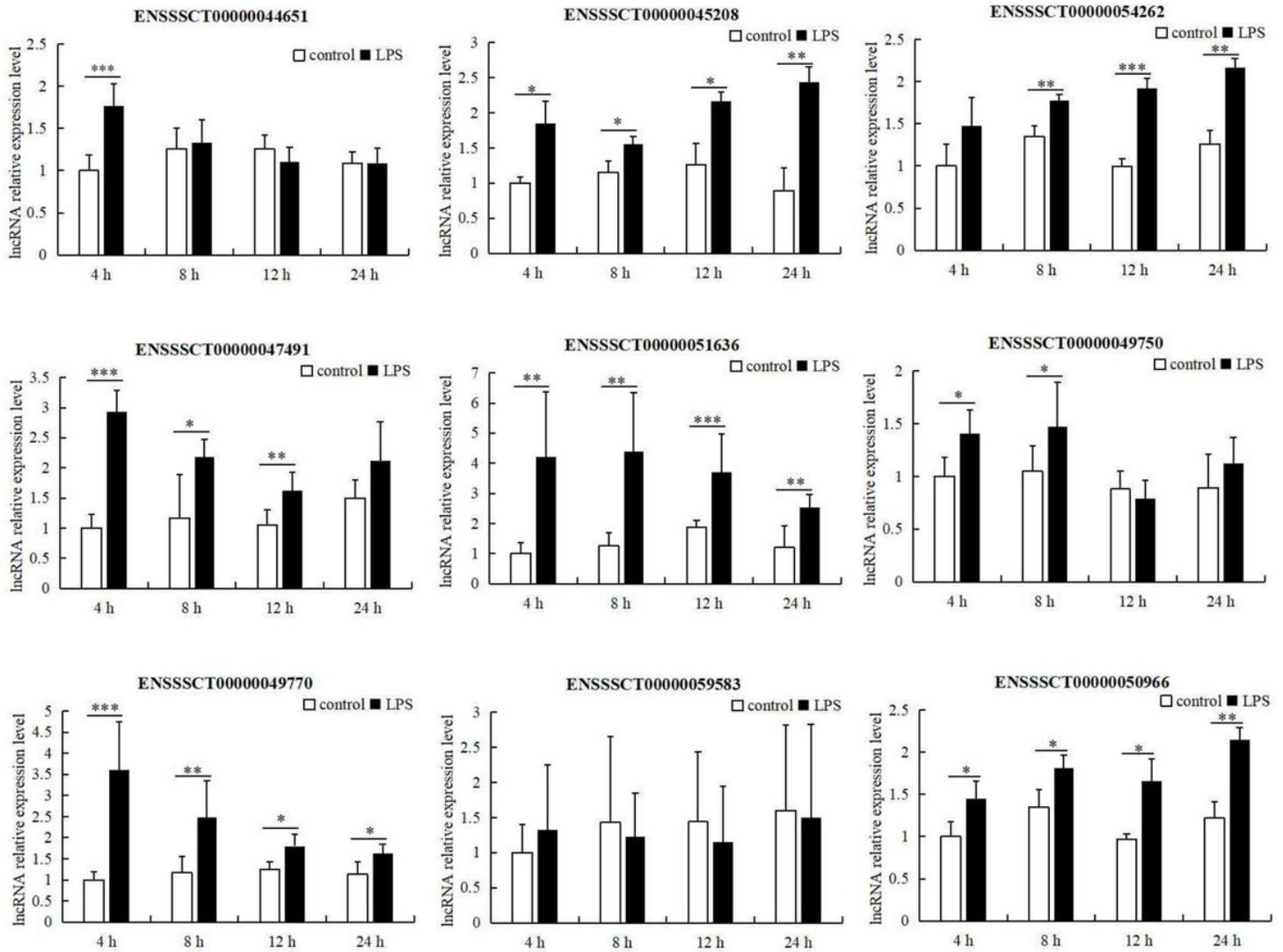


Figure 6

qRT-PCR validations of the 9 selected known lncRNAs in porcine PBMCs at 4, 8, 12 and 24 h after treatment with or without LPS. The data represent the mean \pm SD. N=3. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.

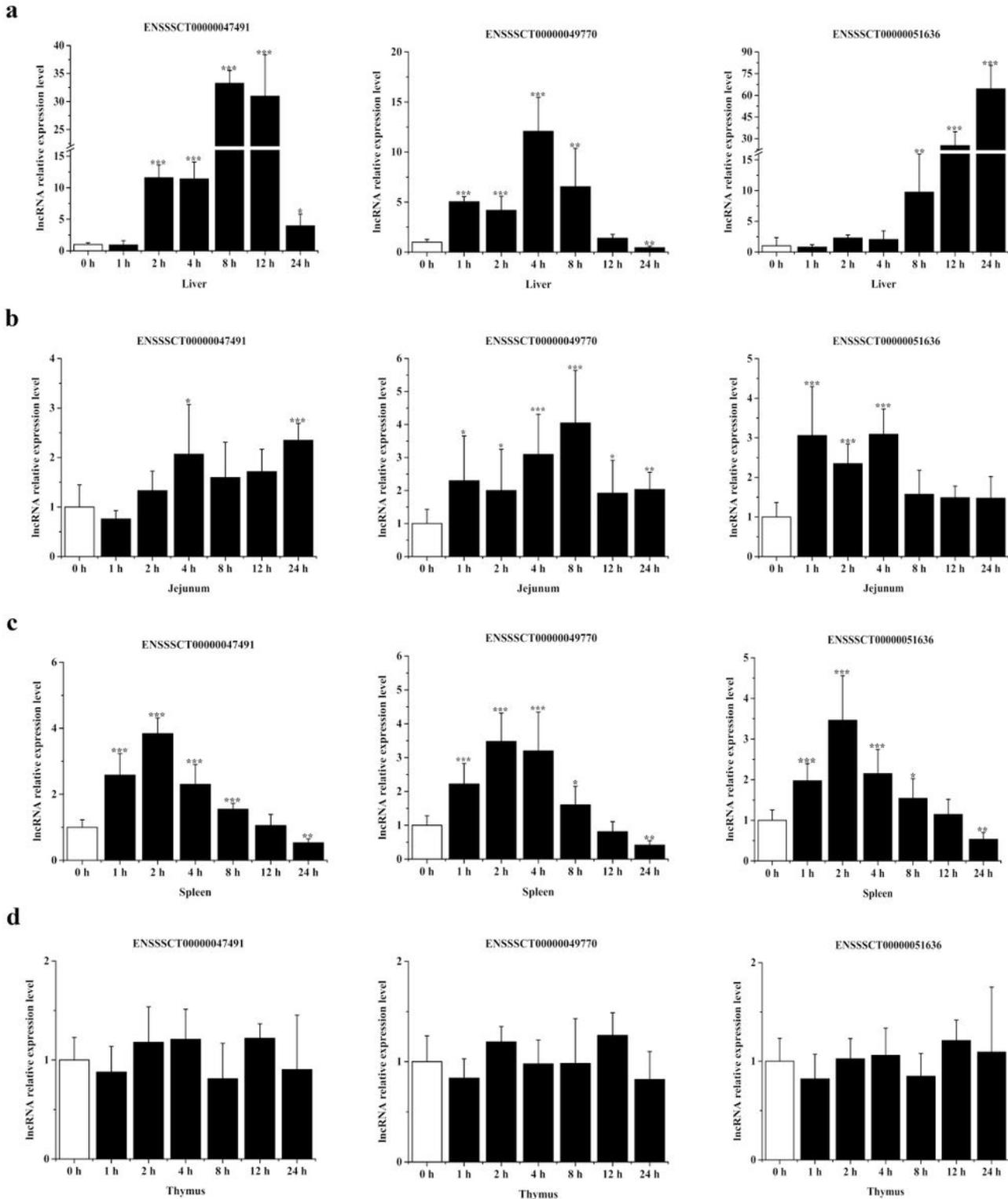


Figure 7

The expression changes of lncRNAs (ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636) in various tissues of piglets challenge with LPS at different times. The qRT-PCR results of the three lncRNAs in liver (a), spleen (b), jejunum (c) and thymus (d) of the piglets challenged with LPS at 0, 1, 2, 4, 8, 12, 24 h. The data represent the mean \pm SD. N=6. *P < 0.05; **P < 0.01; ***P < 0.001 vs. 0 h.

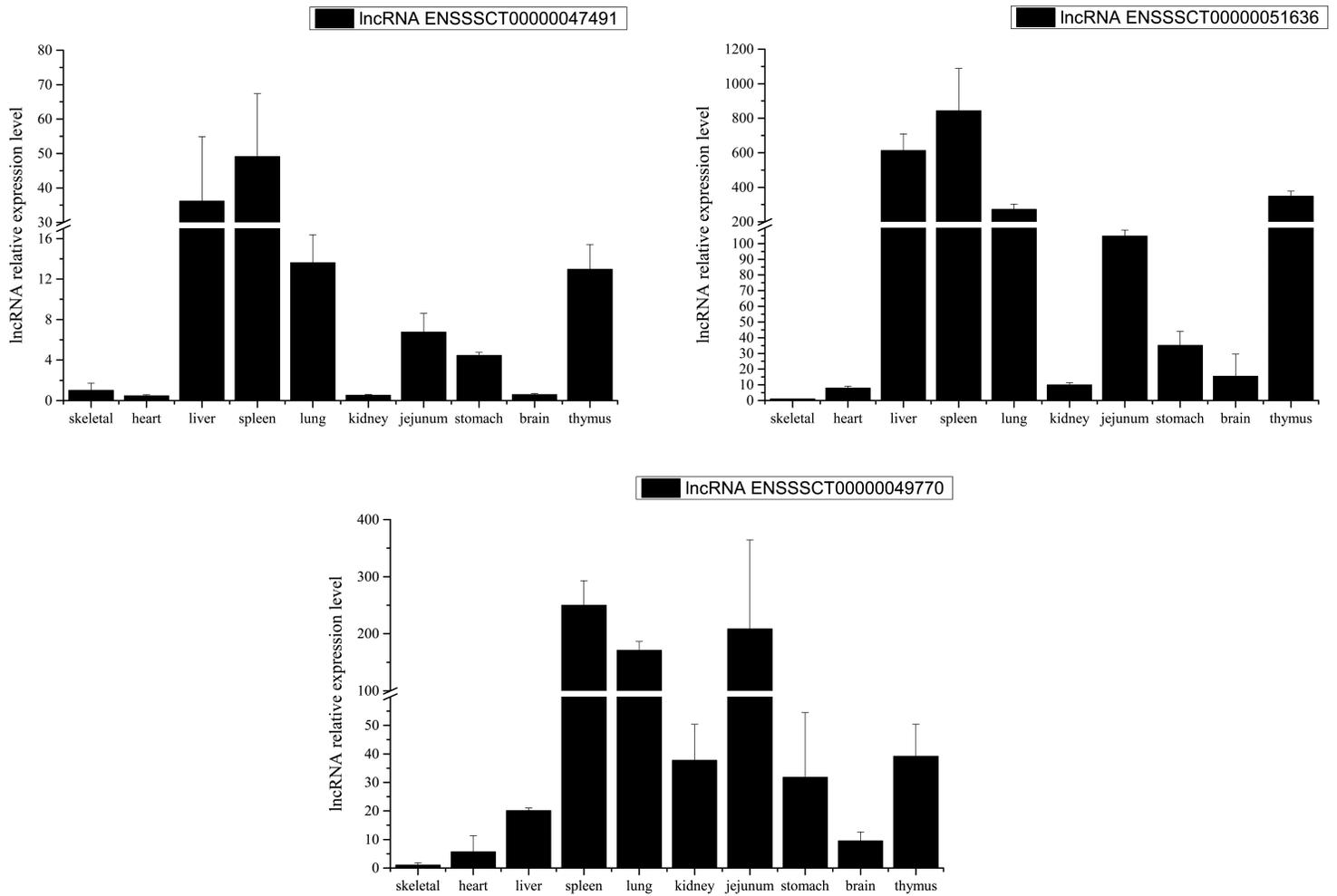


Figure 8

The expression pattern of the three lncRNAs (ENSSSCT0000004749, ENSSSCT00000049770 and ENSSSCT00000051636) in porcine various tissues. The data represent the mean \pm SD. N=2.

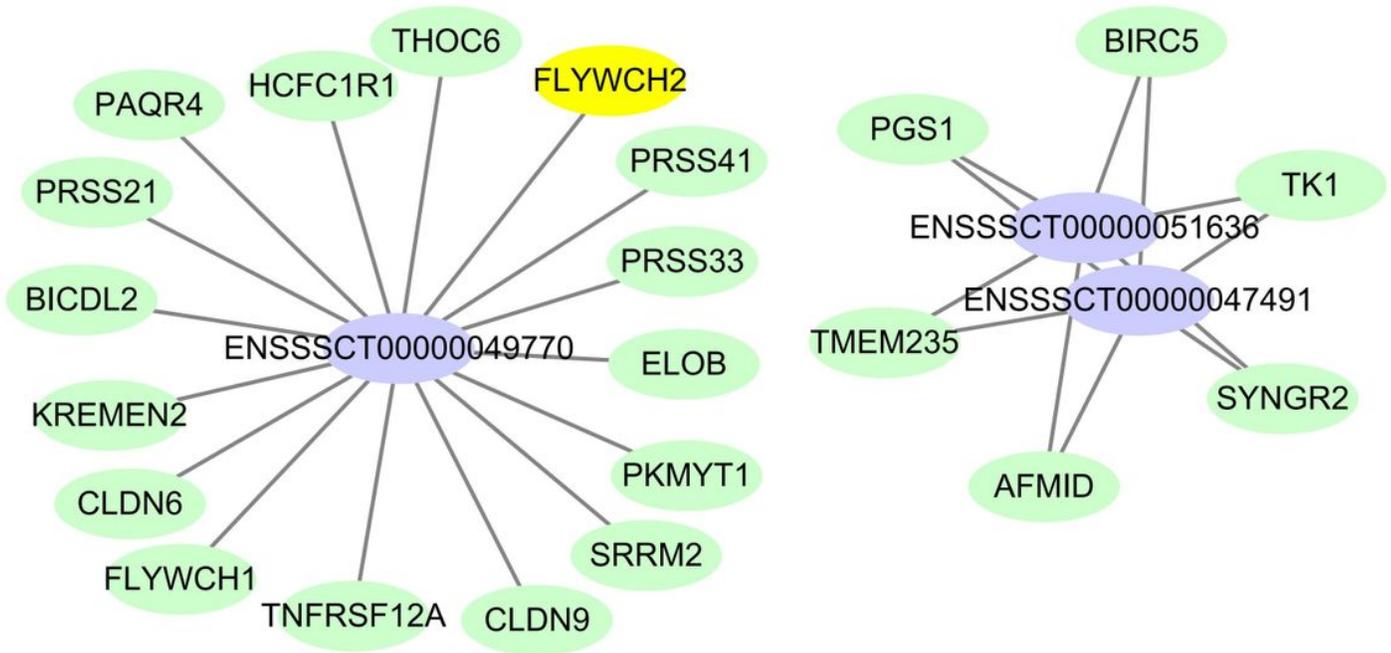


Figure 9

Figure 9

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