Original Article

Molecular Characterization and Expression Analysis of miR-29a in Porcine Cells and Porcine Reproductive and Respiratory Syndrome Virus Infected Peripheral Blood Mononuclear Cells

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Abstract

MicroRNAs are a group of small non-coding RNA that are involved in posttranscriptional regulation through target mRNA degradation or inhibition of protein synthesis. The objective of this present study was to characterize and determine expression level of porcine miR-29a in various tissues. Our results indicated that porcine miR-29a shared high conserved sequence with miR-29a from other organisms. Furthermore, *in silico* analysis suggested that the seed region of porcine miR-29a partially matched IFN-γ mRNA. RT-PCR analysis indicated that miR-29a expressed in various tissues including lymph node, spleen, peripheral blood mononuclear cells (PBMCs) and PK-15 cells. Expression profiling of miR-29a in PRRSV-infected PBMCs *in vitro* revealed an upregulation at 24 and 48 h. Moreover, expression analysis of miR-29a showed higher transcripts in both low pathogenic and highly pathogenic-PRRSV infected pigs. Together, all of these results revealed that porcine miR-29a was highly conserved with miR-29a from other organisms and miR-29a transcripts increased in PRRSV-infected PBMCs both *in vitro* and *in vivo*. Additional studies of the role of miR-29a will provide valuable information on virus: host interaction and could be applied for PRRS control in the future.

Keywords: immunity, miR-29a, porcine, PRRSV

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an important viral disease affecting pigs worldwide. The causative agent is PRRS virus, which is a single stranded, positive sense RNA virus in the family of *Arterivirdae* (Frossard et al., 2013). Presently, two different genotypes of PRRSV, genotype I (European) and genotype II (North American), have been characterized since its initial outbreak. To avoid host immune response, PRRSV employs multiple strategies to inhibit host innate and adaptive immune response. For example, PRRSV non-structural proteins can interfere with type I IFN production (Sun et al., 2012[b]). Moreover, the virus nucleocapsid protein can stimulate regulatory T cell differentiation and IL-10 production (Wongyanin et al., 2012). All of these mechanisms are responsible for poor adaptive immune response as suggested by delay neutralizing antibody production and less IFN-γ secreting cells in PRRSV-infected pigs (Labarque et al., 2000; Díaz et al., 2005). Such an inefficient immune response allows viral persistent in pigs up to 251 days (Wills et al., 2003).

MicroRNAs are a group of conserved small RNA with 22 nucleotide lengths that participate in the process of posttranscriptional regulation. MiRNAs have been identified across multiple organisms including plants, animals, human as well as viruses (Bartel, 2009). MiRNAs have been associated with a wide variety of biological processes such as oncogenesis, organ development, and host pathogen interaction (Krol et al., 2010). To regulate gene expression, specific sequence located within 5′ untranslated region (UTR) of miRNA binds to target mRNA, leading to mRNA degradation or inhibition protein translation (Bartel, 2009). Recent studies clearly suggested that both innate and adaptive immunity were finely regulated by multiple miRNAs (Chen et al., 2013). Among these, members of the miR-29 family have been shown as important regulators of diverse immunological pathways (Liston et al., 2012; Brain et al., 2013). In particular, miR-29 targeted IFN-γ production by directly inhibiting IFN-γ mRNA (Ma et al., 2011; Savan et al., 2011) or indirectly regulating two transcription factors Eomes and T-bet (Steiner et al., 2011).

To date, there is no available information on miR-29a expression during viral infection in porcine cells. Based on the conserved sequence of miR-29a among different species and its critical role in immune system, it is interesting to characterize porcine miR-29a and determine expression level of miR-29a in normal porcine tissues and cell lines. In addition, analysis of miR-29a expression in PBMCs was investigated during PRRSV infection both *in vitro* and *in vivo*. Distinct expression pattern of miR-29a may provide valuable information to the role of this microRNA in pig.

Materials and Methods

Characterization of porcine miR-29a: To characterize porcine miR-29a, sequences of miR-29a were retrieved from miRbase (www.miRbase.org) and NCBI (www.ncbi.nlm.nih.gov). The *Sus scrofa* miR-29a (NR_038524) sequence was compared with miR-29a from other organisms including *Homo sapiens* (NR_029503), *Rattus norvegicus* (NR_031836), *Mus musculus* (NR_029744), *Bos taurus* (NR_031267), *Danio rerio* (NR_030039), and *Gallus gallus* (NR_031391). The secondary structure of precursor miRNA was predicted by RNA Folding Form, Mfold program (Zuker, 2003). Comparisons of miR-29a precursor and mature sequence among species were examined by multiple sequences alignment using ClustalW2 program, European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI).

PBMC preparation and expression of miR-29 in normal cells and tissues: Tissues including spleen and mesenteric lymph nodes were collected from three healthy pigs. PBMC were isolated from heparinized whole blood samples using Ficoll-Hypaque Plus (GE healthcare, USA) density gradient centrifugation. Blood samples were initially diluted in sterile 1X phosphate buffer saline (PBS) (PBS : whole blood = 1 : 1). The diluted blood was overlayed on Ficoll-Hypaque Plus (diluted blood : Ficoll = 10 : 3) in 15 ml conical tube and centrifuged at 2500 rpm for 20 min at 20°C. After centrifugation, PBMCs layer was carefully collected into a new conical tube. To remove contaminated red blood cells, samples were incubated with 5 ml of sterile Red Blood cell (RBC) lysis solution

Table 1 All Primers used in this study

Target	Primer name	Primer sequence (5' – 3')	Tm (°C)	Size (bp)	GenBank Accession number
miR-29a	miR29A_F	CCCCTTAGAGGATGACTGAT	57.3	86	NR_038524.1
	miR29A_R	CCCCAATCATTATAACCGAT	53.2		
GAPDH	GAPDH_F	AGGTCATCCATGACAACTTCGGCA	72.5	155	AF017079
	GAPDH_R	AGCACCAGTAGAAGCAGGGATGAT	67.9		
β-actin	ACTB_F	GGACTTCGAGCAGGAGATGG	66.2	233	AY550069.1
	ACTB_R	GCACCGTGTTGGCGTAGAGG	70.1		

on ice for 3 min. The reaction was terminated by filling PBS and centrifuged at 2500 rpm for 5 min at 4°C. PBMCs were collected and resuspended with RPMI medium for further experiment.

Total RNA extraction and cDNA synthesis: Porcine cells and tissues were harvested from healthy pigs. The animal use protocol was approved by Chulalongkorn University Animal Care and Use committee, approval number 13310019. Total RNA was extracted by using Trizol solution (Invitrogen, USA) following manufacturer's instructions. Subsequently, RNA was treated with DNase I (Promega) to remove contaminated DNA. Total RNA concentration and quality were examined by NanoDrop spectrophotometer 2000 (Thermo Scientific, USA). To synthesize the first strand cDNA, five hundred nanogram of total RNA in each group was reverse transcribed by Superscript III (Invitrogen, USA) and Oligo dT primer (Promega, USA).

Polymerase Chain Reaction (PCR): To study miR-29a expression in porcine tissues and cells, 2 µl of cDNA was amplified with Taq DNA polymerase (Thermo scientific, USA) with 0.2 µM of forward and reverse primers, 2 mM $MgCl_2$ and 0.2 mM of each dNTP in 20 µl reaction. The PCR conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles (for miR-29a) or 30 cycles (for β-actin) of 95°C for 30 sec, annealing at 55°C (for miR-29a) or 58°C (for β-actin) for 30 sec and then extension at 72°C for 30 sec. The final extension was performed at 72°C for 7 min. The specific PCR products were determined on low melting temperature Nusieve® 3 : 1 agarosegel (Lonza, USA). Primer sequences including GAPDH (Zhang et al., 2012), β-actin (Sun et al., 2012[a]) and miR-29a are shown in Table 1. For qRT-PCR, 2µl of cDNA was amplified in 20 µl reaction with iTaq Universal SYBR green master mix (Biorad, USA). The qRT-PCR protocol was performed according to our recent publication (Nantakhruea et al., 2013). Expression level of miR-29a and β-actin was calculated by $2^{-\Delta\Delta Cq}$ method for relative quantification (Livak and Schmittgen, 2001).

Expression of miR-29a in PRRSV-infected PBMCs: In this study, PBMCs were plated in RPMI medium at concentration of $1x10^6$ cells per well in 96 well plate. After plating, the cells were incubated with low pathogenic local Thai PRRSV isolates, 01NP1 (North American genotype, GenBank accession number: DQ056373.1) at 0.1 multiplicity of infection (MOI). PBMCs were maintained at 37°C with 5% CO_2 for 24 and 48 h. To determine miR-29a expression, total RNA was collected and processed for qRT-PCR as previously described (Nantakhruea et al., 2013).

Expression of miR-29a in PRRSV-infected pigs: Piglets were divided into three groups including control, pigs challenged with low pathogenic PRRSV (LP-PRRSV) strain 01NP1, and pigs challenged with highly pathogenic PRRSV (HP-PRRSV) strain 10PN01 at concentration of 10^5 $TCID_{50}$/ml. All piglets were inoculated via intranasal route with either cell culture medium, LP-PRRSV or HP-PRRSV. After 10 days post PRRSV inoculation, heparinized whole blood was collected from PRRSV-infected and non-infected pigs. PBMCs were separated from each blood sample by Ficoll-Hypaque density gradient centrifugation. Expression of miR-29a was examined using RT-PCR. Plasmid containing miR-29a fragment (pGEM-miR29a) was used as a positive control in the PCR reaction (Nantakhruea et al., 2013).

Statistical analysis: The paired sample *t* test was used for statistical analysis. Data were analyzed with GraphPad Prism software version 5.01 (GraphPad, San Diego, CA). A *p*-value ($p<0.05$) indicated statistical significance.

Results

Secondary structure and multiple sequence alignments of miR-29a: Sequences of precursor and mature miR-29a from each species including pig (*Sus scrofa*), human (*Homo sapiens*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), cow (*Bos taurus*), zebra fish (*Danio rerio*) and chicken (*Gallus gallus*) were compared. In Table 2, the predicted secondary structure of miR-29a demonstrated that the pig miR-29a contained similar hairpin conformation with miR-29a from the other species. Furthermore, the analysis of mature miR-29a sequence indicated that the porcine mature miR-29a shared more than 95% sequence similarity with the other species (data not shown). Intriguingly, the seed region at the end of 5′ UTR of porcine mature miR-29a contained conserved 7 nucleotide sequences of AGCACCA which is similar to the seed region of mature miR-29a from the other species (Fig 1a). In addition, comparison of the porcine miR-29a with IFN-γ mRNA indicated 7 bases complementary binding on IFN-γ mRNA with one mismatch (Fig 1b).

Expression of miR-29a in normal porcine cells and tissues: To determine expression level of miR-29a in normal porcine tissues, total RNA were extracted

a

Sus scrofa	3′ - AUUGGCUAAAGUCUACCACGAUC - 5′
Homo sapiens	3′ - AUUGGCUAAAGUCUACCACGAU - -5′
Rattus norvegicus	3′ - AUUGGCUAAAGUCUACCACGAU - -5′
Mus musculus	3′ - AUUGGCUAAAGUCUACCACGAU - -5′
Bos taurus	3′ - AUUGGCUAAAGUCUACCACGAUC - 5′
Danio rerio	3′ - AUUGGCUAAAGUUUACCACGAU - -5′
Gallus gallus	3′ - - UUGGCUAAAGUUUACCACGAU - -5′

b

MiR-29a	3′ - AUUGGCUAAAGUCUACCA CGAUC - 5′
	| | | | | | | |
IFN-γ mRNA	5′ - CAGAGGT TCC TAAATGGTAGCTCT - 3′

Figure 1 Comparison of mature miR-29a from pig and other species. Underlines indicated conserved seed region that binds complementary to target mRNA (Fig 1a). Partial alignment of the seed region of porcine miR-29a with mRNA sequence of IFN-γ (accession number NM_213948.1) (Fig 1b)

Table 2 Secondary structure of precursor miR-29a from various organisms

Species	Secondary structure of miR-29a
Sus scrofa	UUAGAGG UUU C UCAAU CCCC AUGACUGAUUUC UGGUGUU AGAG \ GGGG UAUUGGCUAAAG ACCACGA UCUU A UUAGUAA UCU - UUAAU
Homo sapiens	UUU C UCAAU AUGACUGAUUUC UGGUGUU AGAG \ UAUUGGCUAAAG ACCACGA UCUU A UCU - UUAAU
Rattus norvegicus	A UUAGAGG UUU C UCAAU CCCC AUGACUGAUUUC UGGUGUU AGAG \ GGGG UAUUGGCUAAAG ACCACGA UCUU A A UUAGUAA UCU - UUAAG
Mus musculus	A UUAGAGG UUU C UCAAU CCCC AUGACUGAUUUC UGGUGUU AGAG \ GGGG UAUUGGCUAAAG ACCACGA UCUU A A UUAGUAA UCU - UUAAG
Bos taurus	UUU C UCAAU AUGACUGAUUUC UGGUGUU AGAG \ UAUUGGCUAAAG ACCACGA UCUU A UCU - UUAAU
Danio rerio	U CCCUCAUCUCUCUCUCUCUC CCAAACG CUU U GUCCCA GAAGA UCCCCA AUGACUGAUUUC UGGUGCU AGA \ CUUCU AGGGGU UAUUGGCUAAAG ACCACGA UCU U A UAACU - - - - - - - - - - - - - - - CAGUAA- UUU - ACUGUC
Gallus gallus	A UUUAGAGG UUU C UCAAU CCCC AUGACUGAUUUC UGGUGUU AGAG \ GGGG UAUUGGCUAAAG ACCACGA UCUU A A UUAGUGA - UUU - UUAUA

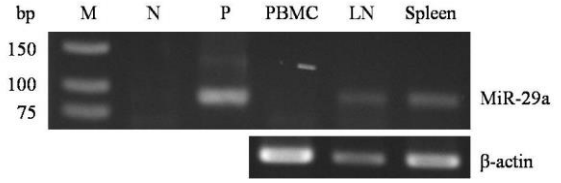

Figure 2 Expression of miR-29a in normal porcine tissues including PBMCs, mesenteric lymph nodes and spleen. β-actin was used as internal control gene. M: 10 bp marker, N: no template control, P: positive control (pGEM-miR29a), PBMC: peripheral blood mononuclear cells, LN: mesenteric lymph nodes

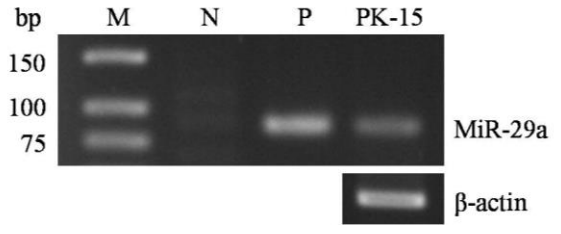

Figure 3 RT-PCR analysis of miR-29a expression in PK-15 cell line. M: 10 bp marker, N: no template control and P: positive control (pGEM-miR29a)

from mesenteric lymph nodes, spleen and PBMCs. RT-PCR analysis revealed a single specific product of miR-29a at 86 bp. Interestingly, miR-29a was highly expressed in mesenteric lymph nodes and spleen, whereas the expression of miR-29a was low in PBMCs (Fig 2). In addition to the porcine tissues, we examined miR-29a expression in porcine kidney cell line (PK-15). As shown in Figure 3, miR-29a transcript could be detected in normal PK-15 cells. These findings indicated that miR-29a was expressed in both porcine immune and non-immune cells.

Expression of miR-29a in PRRSV-infected PBMCs: To determine the expression pattern of miR-29a during PRRSV infection, PBMCs were isolated from PRRSV-free pigs and plated in 96 well plate. The cells were then inoculated with LP-PRRSV at MOI 0.1 for 24 and 48 h. Expression of miR-29a was investigated by qRT-PCR method and normalized against GAPDH control gene. After PRRSV infection, we found that the expression of miR-29a increased 5.2 and 4.1 folds at 24 and 48 h, respectively, with higher expression at 24 h post infection (Fig 4). However, there was no statistical significant between control and PRRSV-infected groups ($p < 0.05$).

Expression of miR-29a in PRRSV-infected pigs: Based on the expression of miR-29a in PRRSV-

infected PBMCs *in vitro*, it is interesting to investigate level of miR-29a in PBMCs from PRRSV-infected statistical significance among the control, LP-PRRSV and HP-PRRSV infected animals ($p<0.05$), we observed an increasing trend of miR-29a in both LP-PRRSV and HP-PRRSV infected pigs (Fig 5a). Specifically, the pigs infected with LP-PRRSV and HP-PRRSV has miR-29a transcript increase at 2.2 and 1.8 folds, respectively (Fig 5b).

Discussion

MicroRNAs are evolutionary conserved, non-coding RNAs that negatively regulates target gene expression at posttranscriptional process. To date, hundreds of microRNAs have been characterized in pigs during normal biological process and pathogen response (Xie et al., 2011; Podolska et al., 2012). In this study, we characterized porcine miR-29a and determined its expression level during normal condition and viral infection. Our results showed that porcine miR-29a shared high conserved sequence with miR-29a from other organisms (Xie et al., 2011; Chen et al., 2012). Furthermore, the sequence of porcine miR-29a seed region shared 100% sequence similarity to miR-29a seed region from human, mouse, rat, cow, chicken and fish. In human and mice, the seed region of miR-29a directly targeted 3'UTR of IFN-γ mRNA, leading to low IFN-γ production (Ma et al., 2011; Savan et al., 2011). Moreover, miR-29a regulated IFN-γ production in helper T cells through indirectly targeting two transcription factors Eomes and T-bet (Steiner et al., 2011).

Following the characterization of porcine miR-29a, the expression pattern of miR-29a was investigated in various porcine tissues (mesenteric lymph nodes, spleen and PBMCs) and PK-15 cell line. Our findings suggested that miRNA-29a expressed in all normal porcine tissues and cell lines with higher expression in spleen and lymph nodes than PBMCs. Similar to this finding, different expression patterns of miRNA in mice and human were reported in various tissues and correlated with function and disease pathogenesis (Lagos-Quintana et al., 2002; Liang et al., 2007).

One of the important viral diseases affecting pig production is PRRS (Neumann et al., 2005). To establish infection, PRRSV uses multiple strategies to suppress pig immune response including the delayed and weak IFN-γ production (Dotti et al., 2013). Based on the sequence similarity with other organisms and expression pattern of porcine miR-29a in stimulated PBMC (Nantakhruea et al., 2013), it is interesting to examine miR-29a transcripts in PBMC inoculated with PRRSV. At 24 and 48 h post infection, we found an upregulation of miR-29a in PRRSV-infected PBMC relative to uninfected control. Similar to *in vitro* observation, expression analysis of miR-29a showed higher miR-29a transcript in both LP and HP-PRRSV infected animals. Although both *in vitro* and *in vivo* data did not provide any statistical significance, we still observed similar trend of upregulation of miR-29a in both studies. In addition, our results revealed higher expression of miR-29a in pigs infected with LP-PRRSV than pigs infected with HP-PRRSV. Therefore, it is intriguing to note that different patterns of miR-29a might lead to different immunological outcome between diverse strains of PRRSV infection (Han et al., 2013; Zhang et al., 2013). In conclusion, our results revealed that porcine miR-29a was highly conserved with miR-29a from other organisms and miR-29a transcripts were expressed in normal porcine cells and tissues. Moreover, expression analysis of miR-29a suggested that miR-29a increased in PRRSV-infected PBMCs both in *vitro* and *in vivo.* A more detailed analysis of target gene expression should be examined to better understand the role of miR-29 during PRRSV infection. These data provide valuable information on PRRS virus: host interaction and could be applied for PRRSV control in the future.

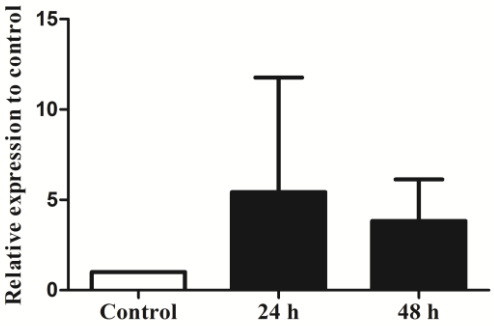

Figure 4 MiR-29a expression in PRRSV-infected PBMCs. PBMCs were incubated with PRRSV for 24 and 48 h. Expression of miR-29a was determined by qRT-PCR and normalized to GAPDH gene. The relative fold change of miR-29a in PRRSV-infected cells was shown as the relative to control

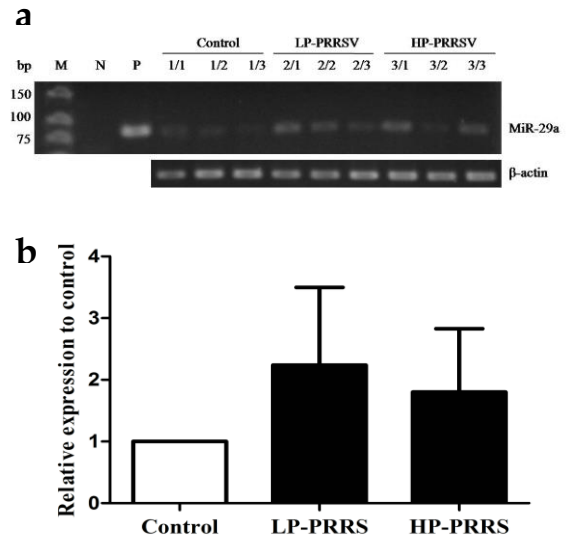

Figure 5 Expression of miR-29a in PBMCs isolated from PRRSV-infected pigs (Fig 5a). Relative fold change of miR-29a was normalized to β-actin gene (Fig 5b). LP-PRRS : low pathogenic PRRS virus infection, HP-PRRS : highly pathogenic PRRS virus infection, control : non-infected PBMC. The β-actin gene was used as an internal control. M : 10 bp marker, N : no template control, P : positive control (pGEM-miR29a)

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บทคัดย่อ

ลักษณะทางโมเลกุลและระดับการแสดงออกของ miR-29a ในเซลล์สุกร และเซลล์เม็ดเลือดขาวที่ติดเชื้อไวรัสพีอาร์อาร์เอส

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ไมโครอาร์เอ็นเอเป็นอาร์เอ็นเอขนาดเล็กที่ควบคุมการแสดงออกของยีนผ่านการทำลายเอ็มอาร์เอ็นเอหรือยับยั้งการสร้างโปรตีน วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาลักษณะทางโมเลกุลและระดับการแสดงออกของ miR-29a ในเนื้อเยื่อสุกร การศึกษาพบว่าลำดับเบสของ miR-29a สุกรมีความเหมือนในระดับที่สูงกับ miR-29a ในสิ่งมีชีวิตอื่น นอกจากนี้การวิเคราะห์ด้วยโปรแกรมคอมพิวเตอร์พบว่าลำดับเบสบริเวณ seed region ของ miR-29a สุกรสามารถจับได้บางส่วนกับอินเตอร์เฟอรรอนแกมม่าเอ็มอาร์เอ็นเอ การศึกษาด้วยเทคนิคอาร์ทีพีซีอาร์พบว่า miR-29a มีการแสดงออกในเนื้อเยื่อ ต่อมน้ำเหลือง ม้าม เม็ดเลือดขาว และเซลล์ PK-15 การแสดงออกของ miR-29a ในเซลล์เม็ดเลือดขาวที่ติดเชื้อไวรัสพีอาร์อาร์เอสมีปริมาณเพิ่มขึ้นที่ 24 และ 48 ชั่วโมง การแสดงออกยังเพิ่มขึ้นในสุกรที่ติดเชื้อไวรัสพีอาร์อาร์เอสทั้งสายพันธุ์ไม่รุนแรงและสายพันธุ์ที่รุนแรง จากผลการทดลองทั้งหมดแสดงให้เห็นว่า miR-29a สุกรมีความคล้ายคลึงกับ miR-29a ของสิ่งมีชีวิตชนิดอื่น และระดับการแสดงออกของ miR-29a มีปริมาณเพิ่มขึ้นในเซลล์เม็ดเลือดขาวที่ติดเชื้อพีอาร์อาร์เอสในหลอดทดลองและในสุกรมีชีวิต การศึกษาเพิ่มเติมเกี่ยวกับหน้าที่ของ miR-29a จะช่วยอธิบายปฏิสัมพันธ์ระหว่างไวรัสและโฮสต์ ซึ่งอาจนำไปสู่การพัฒนาวิธีป้องกันโรคพีอาร์อาร์เอสในอนาคต

คำสำคัญ: ภูมิคุ้มกัน miR-29a สุกร ไวรัสพีอาร์อาร์เอส

ภาควิชาจุลชีววิทยาและวิทยาภูมิคุ้มกัน คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพฯ 10900
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