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Research paper

Functional characterization of porcine LSm14A in IFN-β induction

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ABSTRACT

Human LSm14A has recently been found as a processing body-associated sensor of intracellular viral nucleic acids and triggers signaling for type I IFN expression. Here porcine LSm14A (pLSm14A) was cloned from the PK-15 cells. The *pLSm14A* ORF is 1392 bp in length, encoding 463 amino acids. The putative pLSm14A contains a Sm-like domain and two arginine–glycine–glycine (RGG) boxes. The pLSm14A has high identity at the amino acid level to those of bovine, human and mouse (93.5–97.4%) and is transcribed in different tissues of pigs. In HEK293 or Marc-145 cells, pLSm14A was localized in the cytosol as P-body-like dots. Expression of pLSm14A in HEK293 or Marc-145 cells enhanced activities of IFN-β and NF-κB promoters, induced IFN-β transcription, and potentiated poly(I:C)-induced IFN-β promoter activation, indicating that pLSm14A is a potential signal molecule in the IFN-β pathway of pigs. We also found that pLSm14A-induced IFN-β promoter activity was down-regulated by porcine reproductive and respiratory syndrome virus infection in Marc-145 cells. Since pLSm14A is constitutively expressed in virtually all tissues, more research is needed to explore its role in initial phase of viral infections of pigs and its relationship with RIG-I in sensing PAMPs for type I IFN induction.

1. Introduction

The innate immune responses, the first line of host defense against invading pathogens, are activated through the pattern recognition receptors (PRRs) of the host upon recognition of pathogen associated molecular patterns (PAMPs) with the resulting expression of a variety of genes responsible for inflammatory and immune responses (Akira et al., 2006). The PRRs thus far identified include the toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) while PAMPs from microbial sources cover a number of components such as nucleic acids, replicative intermediates, transcription products, LPS and other cell wall components (Akira et al., 2006). Type I interferons, IFN-α and IFN-β, are the major effector cytokines of innate responses of the host to viral and bacterial pathogens (González-Navajas et al., 2012). The signaling network of type I interferon induction involves a number of adaptor proteins, such as MyD88, TRIF, VISA and STING that relay the signals to IRF3, IRF7 and NF-κB via kinases TAK1, TBK1 or IKK (Zhong et al., 2008; Kawai and Akira, 2011; Chen and Jiang, 2013).

LSm14A, also known as RAP55 (mRNA-associated protein 55), is a member of the Sm-like protein family (LSm). It is a component of the mRNA processing bodies (P-bodies) that are present in the cytoplasm as granules involved in RNA metabolism (Yang et al., 2006). Recently, human

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LSm14A was reported as a mediator of IFN-β induction by recognizing synthetic or viral dsRNA and dsDNA via RIG-I, VISA and MITA, thus activating the downstream transcription factors, including interferon regulatory factor-3 (IRF3) and nuclear factor-kB (NF-kB) with induced expression of IFN-β (Li et al., 2012). Although there are quite a number of RAP55 molecules from different species identified (Marnef et al., 2009), few were reported for their putative roles in innate immunity.

To investigate the roles of LSm14A homolog from swine in effecting type I IFN expression, we cloned and characterized the putative porcine LSm14A (pLSm14A) cDNA from PK-15 cells. We demonstrated that pLSm14A localized in the cytosol both in HEK293 and Marc-145 cells and was able to activate IFN-β expression, indicating that pLSm14A is a potential signal molecule in the IFN-β pathway. We also found that pLSm14A-induced IFN-β expression was down-regulated by porcine reproductive and respiratory syndrome virus infection in Marc-145 cells.

2. Materials and methods

2.1. Cell lines and plasmids

The two cell lines (HEK293, a human embryonic kidney cell line and Marc-145, an African green monkey kidney cell line) were cultivated in high glucose Dulbecco's modified Eagle's medium (DMEM)(Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone, South Logan, UT, USA). Vectors used in this study included pEASY-Blunt plasmid (TransGen Biotech Co., Ltd., Beijing, China), pET30a(+) (Novagen, Darmstadt, Germany), pcDNA3.1-5′Flag, pIFN-β-Luc (Dr. Hongbing Shu, Wuhan University, Wuhan, China) that expresses firefly luciferase under the control of the IFN-β promoter, pNifty-Luc (a NF-κB reporter plasmid) and pRL-TK (Promega, Madison, WI, USA).

2.2. Cloning and sequence analysis of porcine LSm14A

BLAST search found a swine sequence (clone THY010088D02, GenBank accession AK351793.1) with high similarity to human LSm14A (GenBank accession NM015578.2) at the N-terminal region. A primer pair was designed on the basis of LSm14A sequences of different species to amplify the coding region of putative porcine LSm14A by reverse transcription PCR from the total RNA extracted from PK-15 cells (Table 1). The purified PCR product was cloned into the pEASY-Blunt plasmid (named as pEASY-LSm14A) for sequencing (Invitrogen, Shanghai, China) and subsequent subcloning.

The amino acid sequences of LSm14A from swine and other species were aligned and phylogenetic tree was constructed using the Clustal W program. Comparisons of the predicted domains, motifs and features of pLSm14A from different species were performed on the Sample Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1).

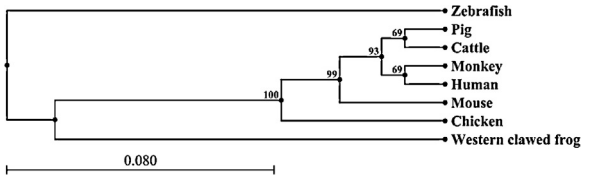

Fig. 1. Phylogenetic analysis of LSm14A molecules from porcine and other species. The sequences were derived from GenBank entries with accession numbers NM015578.2 *Homo sapiens* (human), NM025948.2 *Mus musculus* (mouse), NM001034654.1 *Bos Taurus* (cattle), BC065337.1 *Danio rerio* (Zebrafish), NM213667.1 *Xenopus* (Silurana) *tropicalis* (western clawed frog), NM001012778.1 *Gallus gallus* (chicken), and NM001257954.1 *Macaca mulatta* (monkey).

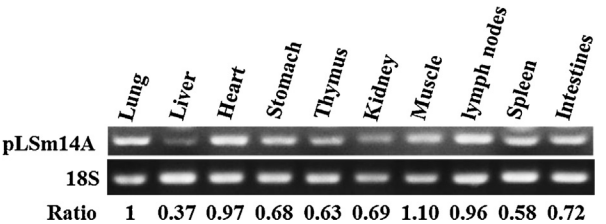

Fig. 2. pLSm14A expression in tissues of a Landrace pig by semi-quantitative RT-PCR.

Table 1
List of primers used in this study.

Primers	Sequence (5′–3′)	Purpose
pLSm14A	F: AGCGGGGGCACCCCTTACATCG R: CTATGCAGCAACTTTGTTGTCCTTCCT	pLSm14A cloning
pLSm14A-euk	F: CGC GGATCC AGCGGGGGCACCCCTTACATCG R: CTAGTCTAGACTATGCAGCAACTTTGTTGTCCTTCCT	pLSm14A eukaryotic expression plasmid constructing
pLSm14A-prok	F: CCGGAATTCATGAGCGG**c**GGCACCCCTTA R: CCAAGCTT**G**CGCCTGTGCTCTTGATTCTCTATTG	pLSm14A prokaryotic expression plasmid constructing
SemiQ	F: TCTCAAGGTCGTTCAAGCCC R: TCGCCTGTGCTCTTGATTCT	Semiquantitative PCR
18S rRNA	F: TTTCGCTCTGGTCCGTCTTG R: TTCGGAACTGAGGCCATGAT	Semiquantitative PCR
IFN-β	F: TAAGCAGCTGCAGCAGTTCCAGAAG R: GTCTCATTCCAGCCAGTGCT	Quantitative PCR
β-actin	F: CGTGCGTGACATCAAAGAGAAG R: CGTTGCCAATAGTGATGACCTG	Quantitative PCR

2.3. Semi-quantitative RT-PCR analysis of LSm14A distribution in porcine tissues

Tissues from a 50-day old healthy Landrace pig, including lung, liver, heart, stomach, thymus, kidney, muscle, mesenteric lymph nodes, spleen and intestines, were dissected and immediately stored in liquid nitrogen. The animal experiment conformed to the ethics with approval by Zhejiang University Experimental Animal Management Committee (No. 2012021). Total cellular RNA was extracted from homogenized tissues using Trizol reagent (Tiangen Biotech Co., Ltd., Beijing, China) and treated with DNAse I (Thermo, Rockford, IL, USA). Tissue mRNA was reversed to cDNA using oligo-d(T)15

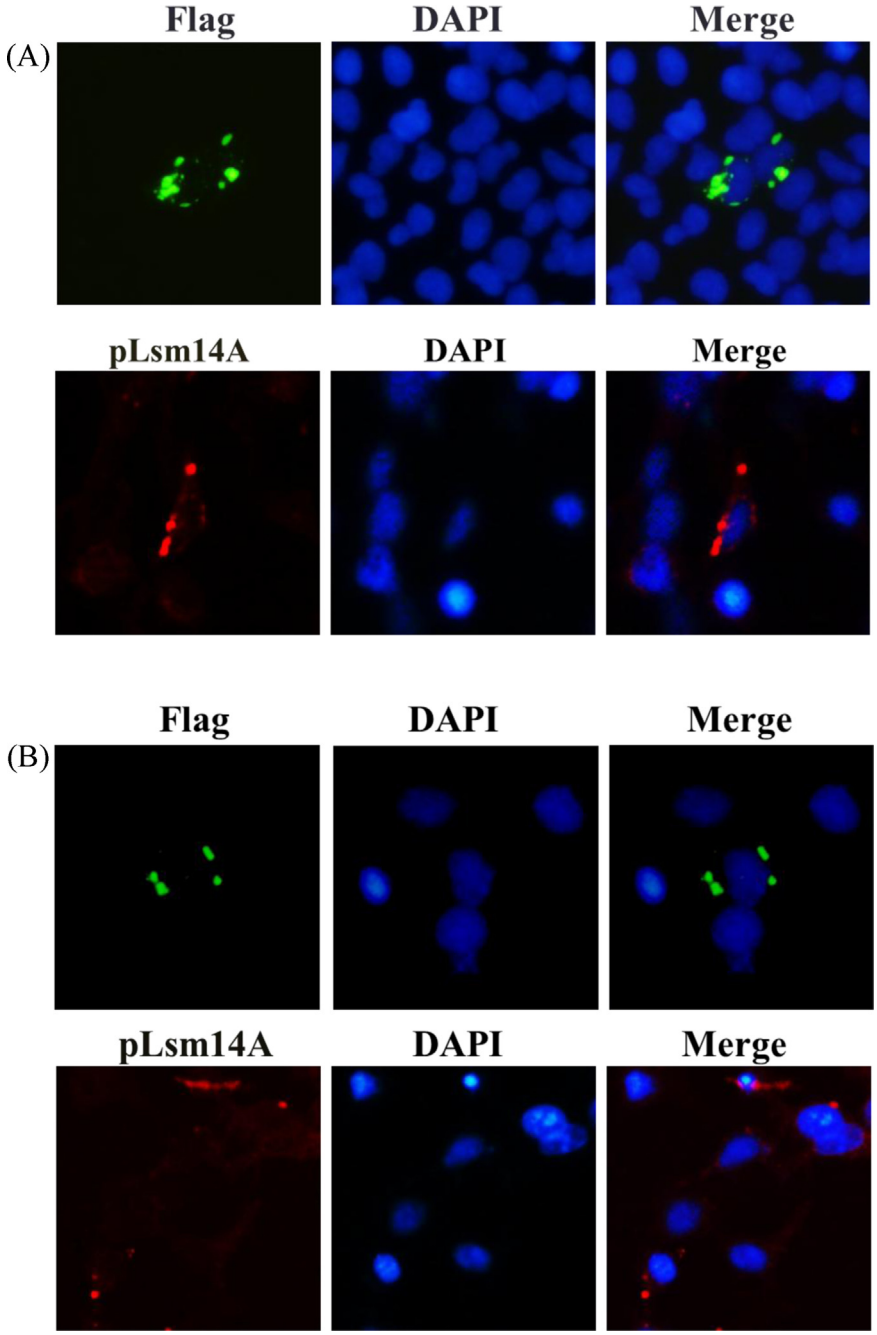

Fig. 3. Localization of pLSm14A in HEK293 (A) and Marc-145 (B) cells. The HEK293 or Marc-145 cells were transfected with pLSm14A expression vector for 24 h before immunofluorescent staining with anti-flag (green) and anti-pLSm14A serum (red). Cellular nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Magnification, 40×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Takara). Semi-quantitative RT-PCR analysis was then performed by using the LSm14A primer pair (Table 1). The 18S rRNA was amplified as an internal control for normalization as previously described (Wang et al., 2008).

2.4. Construction of prokaryotic and eukaryotic expression plasmids

The DNA fragment of pLSm14A containing N-terminal 237aa was PCR-amplified from pEASY-LSm14A using the primer pair pLSm-prok (Table 1). The PCR product was digested with *Eco*RI and *Hind*III (Takara, Dalian, China) and subcloned into the prokaryotic expression vector pET30a(+), resulting in the His tag fusion expression plasmid pET-LSm14A. The recombinant plasmid was then transformed into the host bacteria BL21 (DE3) for protein expression and purification. The purified protein was used to immunize subcutaneously the New Zealand white rabbit (Zhejiang Chinese Medical University Laboratory Animal Center, Hanghzou, China) for preparation of polyclonal antibodies to pLSm14A (animal use approved by Zhejiang University Experimental Animal Management Committee (No. 2012021)).

The full-length ORF of pLSm14A was PCR-amplified from pEASY-LSm14A using the primer pair LSm-euk (Table 1). The PCR product was digested with *BamH*I and *Xba*I (Takara), and subcloned into the mammalian expression vector pcDNA3.1-5′Flag, resulting in pcDNA-pLSm14A.

2.5. pLSm14A expression in eukaryotic cells by immunofluorescence analysis

HEK293 or Marc-145 cells were seeded at a concentration of 3×10^5 cells/well in 30-mm dish containing coverslips (Costar Corning, NY, USA) one day prior to transfection, and incubated until the cells reached approximately 90% confluence. Cells were transfected with 2 μg of pcDNA-pLSm14A using Lipofectamine™ 2000 (Invitrogen, USA) as directed by the manufacturer. At 24 h after transfection, cells were washed twice with PBS (pH 7.2). Coverslips were fixed with 80% acetone at −20 °C for 30 min and incubated with anti-Flag antibody (Sigma, St. Louis, MO, USA) or pLSm14A antiserum at 37 °C for 1 h. Cells were then washed with PBS (pH 7.2) and incubated with FITC-labeled anti-mouse (KPL, Gaithersburg, MD, USA), or Alexa Fluor 555-labeled anti-rabbit antibodies (Invitrogen, USA) at 37 °C for 1 h. Nuclei were stained with 4′,6,-diamidino-2-phenylindole (DAPI). After three times of washing with PBS, coverslips were mounted onto slides and images visualized by fluorescence microscope IX81 (Olympus, Tokyo, Japan).

2.6. Luciferase reporter assays

To examine the effect of pLSm14A on IFN-β or NF-κB promoter activity, HEK293 or Marc-145 cells were seeded and incubated as above. Cells were co-transfected with 1.6 μg/well of pcDNA-pLSm14A or pcDNA3.1-5′Flag (as vector control), 0.4 μg/well of pIFN-β-Luc or pNifty-Luc, and 0.04 μg/well of pRL-TK. Cells were lysed 24 h after transfection with 200 μl of Passive Lysis buffer (Promega). Firefly and renilla luciferase activities in the cell supernatant were measured using the Dual-Luciferase Assay Kit (Promega).

To investigate if pLSm14A would interact with poly(I:C) in inducing IFN-β promoter activity, HEK293 or Marc-145 cells were transfected with pIFN-β-Luc, pRL-TK and increasing amount of pcDNA-pLSm14A (0, 1, 1.5, 2 μg) for 24 h as above. The cells were then transfected with 0.5 μg/well of poly(I:C) for 8 h before luciferase assay or Western blotting. To test if porcine reproductive and respiratory syndrome virus (PRRSV) could affect pLSm14A-induced IFN-β expression, Marc-145 cells were infected with a PRRSV isolate FH1105 (MOI = 1) from a clinical case (North American genotype) for 24 h. The infected cells were

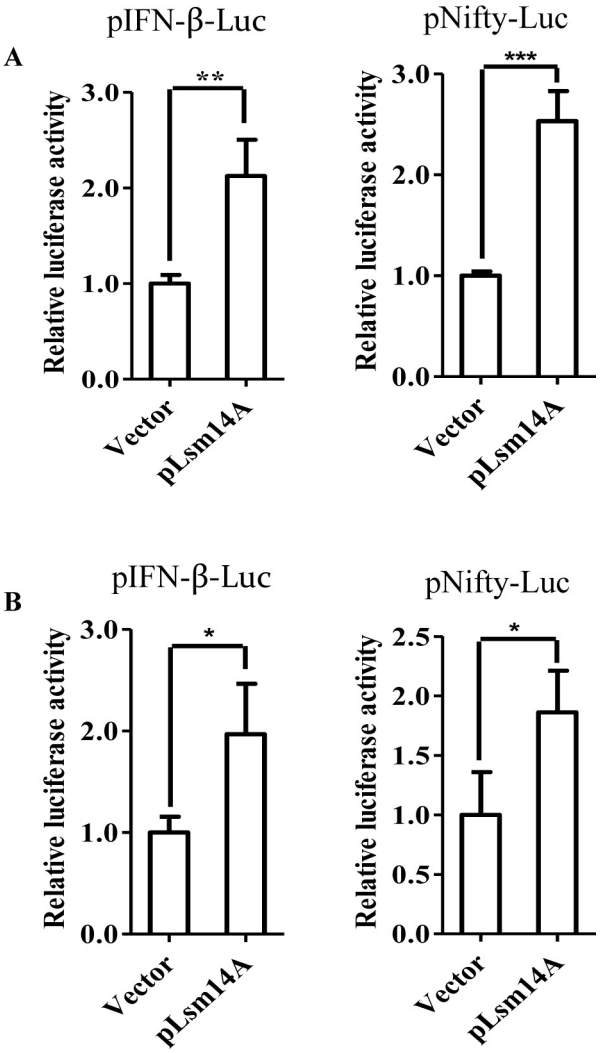

Fig. 4. pLSm14A enhanced activities of IFN-β and NF-κB promoters in HEK293 (A) and Marc-145 (B) cells. HEK293 or Marc-145 cells seeded in 12-well plates were co-transfected with 1.6 μg pcDNA-pLSm14A (or the control vector), 0.04 μg pRL-TK reporter plasmids, and 0.4 μg pNifty-Luc or pIFN-β-Luc. Cells were lysed 24 h after transfection, and examined for luciferase activity. The values were normalized to the renilla activity. Data were presented as fold-changes as compared with the control vector (mean ± SD, $n = 3$) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

then co-transfected with the reporter plasmids (pIFN-β-Luc and pRL-TK, as above) and 1.5 μg pcDNA-pLSm14A for 24 h before luciferase assay. Alternatively, Marc-145 cells were first co-transfected with the reporter plasmids and pcDNA-pLSm14A for 24 h, and then infected with PRRSV (MOI = 1) for 24 h before luciferase assay.

2.7. Transcriptional analysis of IFN-β in cells transiently expressing pLSm14A

The HEK293 or Marc-145 cells were seeded in 12-well plates, incubated as above and transfected with 2 μg pcDNA-pLSm14A or control vector for 24 h. Total RNA was extracted with RNA kit (Tiangen) and treated with DNAse I. cDNA was synthesized with oligo(dT)15, and MLV reverse transcriptase (Promega). Quantitative PCR was performed in triplicate using SYBR Green I dye (Takara) on a Bio-Rad iQ5 system. Primers used for qPCR are shown in Table 1. Results for the target gene were presented after normalization to β-actin. Relative transcript levels were quantified by the $2^{-\Delta\Delta CT}$ method and shown as relative fold changes in comparison with vector control (Livak and Schmittgen, 2001).

2.8. Western blotting

The cell lysates from the above poly(I:C)–pLSm14A interaction experiment were resolved by electrophoresis using 12% SDS-PAGE, and the gel was blotted onto 0.45 μm PVDF membrane (Millipore, Darmstadt, Germany) which was then immuno-blotted with murine monoclonal anti-flag IgG (Sigma) or mouse monoclonal anti-β-actin IgG (MultiSciences Biotech Co., Hangzhou, China) and anti-mouse HRP-labeled antibody (KPL). The protein bands were visualized using enhanced chemiluminescence (Thermo).

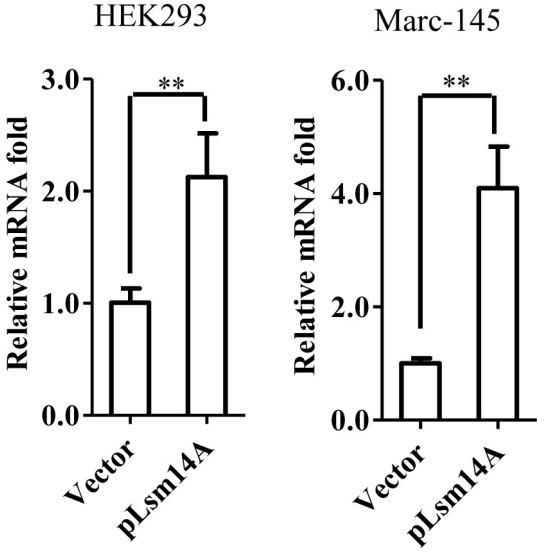

Fig. 5. pLSm14A increased IFN-β gene mRNA transcription in HEK293 and Marc-145 cells. The HEK293 or Marc-145 cells were transfected with 2 μg pcDNA-pLSm14A plasmid (or the control vector) for 24 h for quantitative RT-PCR analysis of IFN-β mRNA transcription. Data were presented as fold-changes, as compared with the control vector (mean ± SD, $n = 3$) (**, $P < 0.01$).

3. Results and discussion

3.1. Cloning and sequence analysis of porcine LSm14A

A 1392 bp gene fragment was obtained as the putative porcine LSm14A (GenBank accession KC769582) using RT-PCR. The full-length ORF that encodes 463 aa was identified by comparing the identified LSm14A of other species. The pLSm14A showed higher identity at the amino acid level to cattle (97.4%), human (97.2%), monkey (96.3%), mouse (93.5%) and chicken (89.8%) than western clawed frog (76.3%) and zebrafish (62.8%). Phylogenetic analysis showed that pLSm14A belonged to the same group together with major mammalian species (Fig. 1). Domain search showed that pLSm14A composes of the

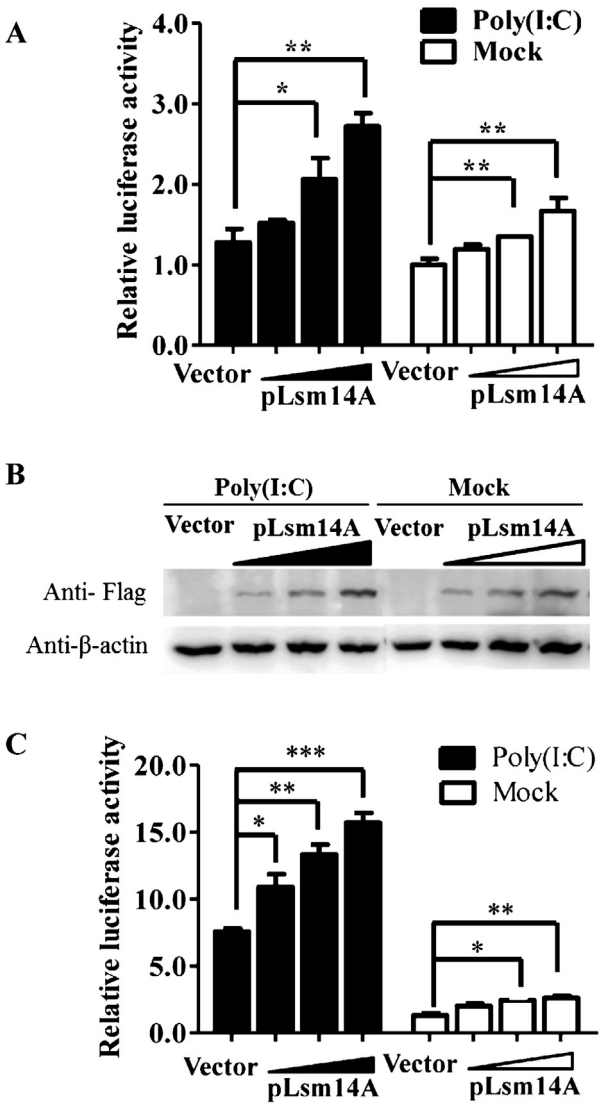

Fig. 6. pLSm14A activated IFN-β promoter in a dose-dependent manner in HEK293 (A) and Marc-145 (C) cells. The HEK293 or Marc-145 cells were transfected with pIFN-β-Luc reporter and increasing amount of pcDNA-pLSm14A (1, 1.5, 2 μg) for 24 h, and then transfected with poly(I:C) for 8 h before luciferase assays or western blot (B). Each datum point represents mean (±SD) of triplicate wells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Sm-like domain (1-52 aa) at its N terminus and the domains involved in accumulation in P-bodies, including the phenylalanine–aspartate–phenylalanine (FDF) domain at aa 286–317, an FFD–TFG box at aa 361–400, and two arginine–glycine–glycine (RGG) boxes at aa 269–285 and aa 404–449) (Yang et al., 2006, Marnef et al., 2009). The Sm-like domain of LSm14A exhibited higher similarity than the other domains or regions in different species from zebrafish to human, and may be involved in binding to mRNA (Yang et al., 2006). However, proteins with RGG boxes could have RNA binding potential that may determine RNA fate from synthesis to decay (Castello et al., 2012). Further research is required to understand if the RGG boxes in porcine or human LSm14A could be involved in RNA binding and turnover.

3.2. pLSm14A mRNA level in different swine tissues and its cellular localization when expressed in heterogenous cells

The pLSm14A transcript was detected in all examined tissues, including lung, liver, heart, stomach, thymus, kidney, muscle, mesenteric lymph nodes, spleen and intestines (Fig. 2). Notably, transcription level was more abundant in the lung, heart, muscle and mesenteric lymph. With the anti-flag antibody and the rabbit anti-pLSm14A serum, we found that expression of pLSm14A in HEK293 or Marc-145 cells was localized in the cytosol as P-body-like dots (Fig. 3). The data on expression patterns in tissues of other animal species are not available. However, human LSm14A has its expression in nearly all tissues localized in the P-bodies and during all developmental stages (Yang et al., 2006; Marnef et al., 2009), which is in contrast with its homologs in *Caenorhabditis elegans* (CAR-1) and *Xenopus* (xRAP55) whose expression is mainly limited to germ cell development and early embryogenesis (Lieb et al., 1998; Audhya et al., 2005). These results might indicate that mammalian LSm14A could play different roles in controlling mRNA translation or turnover, or even in response to viral infections involving both stress granules and P-bodies (White and Lloyd, 2012).

3.3. Porcine LSm14A activated IFN-β signaling

To investigate whether pLSm14A is involved in the type I IFN signaling, HEK293 or Marc-145 cells were transfected with pLSm14A expression plasmid, together with the luciferase reporter plasmids. Over-expression of pLSm14A significantly increased the luciferase activity (Fig. 4), indicating that both IFN-β and NF-κB promoters were activated in the tested cell lines. Increased transcription of IFN-β was also seen from pLSm14A transfected HEK293 and Marc-145 cells (Fig. 5). These results indicate that pLSm14A might act in a way similar to human LSm14A in initiating IFN-β expression (Li et al., 2012).

Then, we used poly(I:C), a synthetic analog of double-stranded RNA (dsRNA) recognized by TLR3 (Alexopoulou et al., 2001) and RIG-I (Kawai et al., 2005), as an inducer in HEK293 and Marc-145 cells transfected with the pLSm14A expression vector to see if there could be interaction between poly(I:C) and pLSm14A in effecting IFN-β signaling. Fig. 6 shows that pLSm14A did potentiate poly(I:C)-triggered IFN-β activation and the response was dose-dependent. Li et al. (2012) showed that poly(I:C) could bind to both RIG-I and LSm14A, individually or simultaneously, and that LSm14A translocates to the peroxisomes where RIG-I and VISA are located (Dixit et al., 2010). Therefore, we suggest that pLSm14A might be acting as a molecular shipper to deliver the PAMPs to RIG-I for sensing and downstream type I IFN signaling via VISA, thus potentiating the poly(I:C)-induced promoter activity.

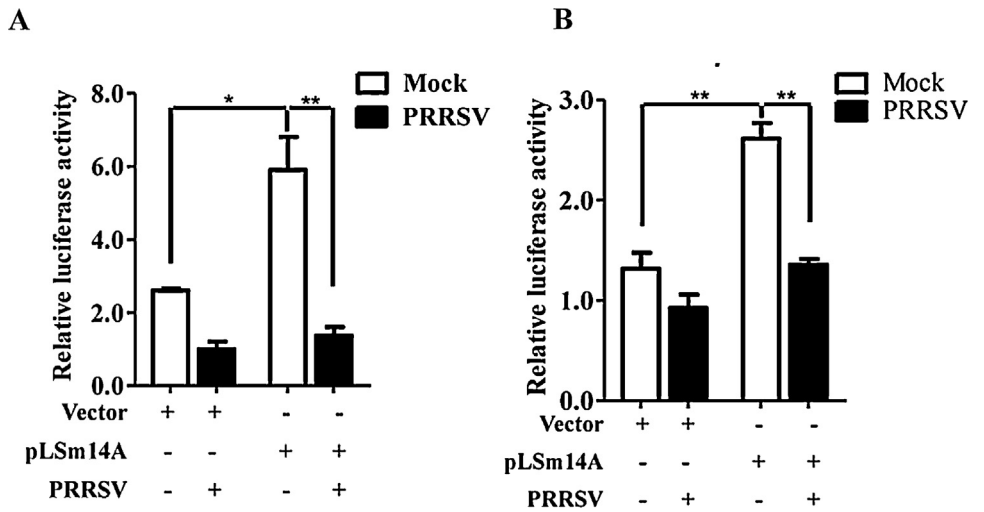

Fig. 7. Inhibition of pLSm14A-mediated IFN-β promoter activity by porcine reproductive and respiratory syndrome virus (PRRSV). (A) Marc-145 cells were infected with PRRSV for 24 h, and then co-transfected with 0.4 μg pIFN-β-Luc, 0.04 μg pRL-TK reporter plasmids and 1.5 μg pcDNA-pLSm14A for 24 h before luciferase assay. (B) Marc-145 cells were first co-transfected with reporter plasmids and pcDNA-pLSm14A as "A" for 24 h, and then infected with PRRSV for 24 h before luciferase assay. Two independent experiments were conducted and showed similar results. Data were presented as fold-changes (mean ± SD) of triplicate wells of one experiment, as compared with the control vector (*, $P < 0.05$; **, $P < 0.01$).

3.4. Porcine reproductive and respiratory syndrome virus infection antagonizes pLSm14A-induced IFN-β promoter activity

We used the porcine reproductive and respiratory syndrome virus (PRRSV), an important swine virus that can evade the innate immune responses (Luo et al., 2008; Sun et al., 2010; Yoo et al., 2010), to see if it is able to block pLSm14A-induced IFN-β signaling in Marc-145 cells. Fig. 7 shows that PRRSV infection could down-regulate the IFN-β-promoter-based luciferase activity when infected either before or after pLSm14A transfection. With the recent findings that human LSm14A translocates to peroxisomes in virus-infected cells and co-localization of VISA with peroxisomes (Li et al., 2012), we postulate that the pLSm14A-RIG-I-VISA pathway could be the target of PRRSV inhibition. This is consistent with a report by Luo et al. (2008) that RRRSV suppresses production of IFN-β primarily by interfering with the IPS-1 (VISA) activation in the RIG-I signaling pathway.

In summary, our results show that the porcine LSm14A molecule is involved in initiating IFN-β signaling like its homologue in humans. Since pLSm14A is constitutively expressed in virtually all tissues, more research is needed to explore its role in initial phase of viral infections of pigs and its relationship with RIG-I in sensing PAMPs for type I IFN induction.

Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgments

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