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Galectin-3 inhibits replication of porcine reproductive and respiratory syndrome virus by interacting with viral Nsp12 *in vitro*

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ABSTRACT

Porcine galectin-3 (GAL3) is a 29-kDa protein encoded by a single gene, *LGALS3*, located on chromosome 1. Here, using a yeast two-hybrid screen of a cDNA library from porcine alveolar macrophage cells (PAMs), we report for the first time that GAL3 interacts with nonstructural protein 12 (Nsp12) of the porcine reproductive and respiratory syndrome virus (PRRSV). Although extensive research has focused on porcine reproductive and respiratory syndrome (PRRS), little is known about the pathogen and host interactions involving individual nonstructural viral proteins, especially Nsp12. Here, we showed that GAL3 interacted with viral Nsp12 following co-transfection of HEK293 cells with GAL3- and Nsp12-expressing plasmids. Additionally, we observed that PPRSV infection led to reduced GAL3 levels during the late phase of infection in both MARC-145 cells and PAMs. Importantly, GAL3 overexpression significantly suppressed the replication of both type 1 and 2 PRRSV strains, whereas knockout of endogenous *LGALS3* in MARC-145 cells significantly increased viral titer and expression of the nucleocapsid protein. These results strongly support a direct inhibitory effect of GAL3 on PRRSV replication, which might contribute to an overall antiviral effect. Furthermore, our findings provide insights into the molecular basis of the role Nsp12 plays in PRRSV pathogenesis.

Porcine reproductive and respiratory syndrome (PRRS) is responsible for reproductive failure in pregnant sows and respiratory illness, particularly in young pigs (Benfield et al., 1992; Snijder et al., 2013). It is also among the most severe infectious diseases threatening the swine industry worldwide for which effective control measures remain scant (Chand et al., 2012; Neumann et al., 2005). Currently, vaccination is the most prevalent method for controlling PRRS; however, commercially available vaccines fail to provide sustainable protection against the disease due to the fast-evolving viral genome and the epidemic variants of the PRRS virus (PRRSV) prevalent in China (Murtaugh and Genzow, 2011; Yoo et al., 2004). A full understanding of the mechanisms associated with regulating PRRSV replication and pathogenesis will provide a solid foundation for identifying alternative measures for controlling multiple circulating PRRSV strains

The causative agent of PRRS, the PRRSV, has a positive-sense, single-stranded RNA genome of ~15 kb that contains at least 10 open reading frames (ORFs) (Chand et al., 2012). ORF1a and ORF1b occupy 80% of the viral genome and encode two important viral replicase polyproteins (pp1a and pp1ab), with pp1ab cleaved into four nonstructural proteins (Nsps) involved in viral-genome transcription and replication (Fang and Snijder, 2010). Among the four putative proteins, Nsp9, Nsp10, and Nsp11 are considered critical contributors to viral replication or virulence (Li et al., 2014c; Zhao et al., 2018). Recently, there has been a rapid increase in studies of virus-host interaction associated with PRRSV pathogenesis (Jin et al., 2017; Li et al., 2014a; Liu et al., 2016; Zhao et al., 2015); however, few attempts associated with Nsp12 have been made. In previous study, Nsp12 was found to induce signal transducer and activator of transcription (STAT) 1 phosphorylation at serine 727 in HEK293 cells (Yu et al., 2013). Heat-shock protein 70 affected viral replication through interaction with viral Nsp12 (Dong et al., 2016). The latest research reported that karyopherin alpha 6 (KPNA6) is required for replication of PRRSV and Nsp12 induced KPNA6 stabilization (Yang et al., 2018). Studies above suggested that, by modulating cellular gene expression, Nsp12 might be involved in PRRSV pathogenesis; therefore, further exploration of interactions between Nsp12 and host cellular proteins is necessary. In the

Abbreviations: Co-IP, co-immunoprecipitation; GAL3, galectin-3; hpi, hours post-infection; hpt, hours post-transfection; IFN, interferon; KO, knockout; MOI, multiplicity of infection; N protein, nucleocapsid protein; Nsp, nonstructural protein; OAS, 2′,5′-oligoadenylate synthetase; PAM, porcine alveolar macrophage; PRRSV, porcine reproductive and respiratory syndrome virus; STAT, signal transducer and activator of transcription; KPNA6, karyopherin alpha 6; WB, western blot

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present study, we performed yeast two-hybrid screening using vHuN4 Nsp12 as a target to elucidate interactions with elements in a cDNA library from porcine alveolar macrophages (PAMs) in order to screen candidate components for novel PRRS-control strategies.

First, we generated a recombinant plasmid, Nsp12-pGBKT7, following amplification of *Nsp12* from the highly pathogenic vHuN4 strain (GenBank accession No. EF635006) and insertion of the amplicon into the yeast plasmid pGBKT7. Yeast two-hybrid screening was performed using Nsp12-pGBKT7 as bait to screen a cDNA library from PAM cells, as previously described (Wang et al., 2012). Sequence alignments were performed using BLAST (https://blast.ncbi.nlm.nih.gov/), allowing determination of the gene (*LGALS3*; GAL3*)* corresponding to the candidate interacting protein (galectin-3; GAL3). To further validate the interaction between GAL3 and Nsp12 in cells, we generated GAL3-c-Myc-pCAGGS and Nsp12-HA-pCAGGS plasmids and co-transfected them into HEK293 cells. The full-length coding sequences of porcine GAL3 and vHuN4 *Nsp12* were amplified and digested with *Eco*RI and *Xho*I (New England Biolabs, Ipswich, MA, USA), and the products were subsequently inserted into the eukaryotic expression vector pCAGGS at sites harboring either a c-Myc or HA tag in order to generate recombinant plasmids. Co-immunoprecipitation (Co-IP) assays were performed at 24-h post-transfection (hpt) and confirmed that GAL3 interacted with viral Nsp12 in HEK293 cells (Fig. 1A). To analyze whether GAL3 co-localized with viral Nsp12, cells were co-transfected with the recombinant plasmids and examined at 24 hpt by confocal immunofluorescence microscopy (Carl Zeiss, Oberkochen, Germany). Results showed that Nsp12 and GAL3 co-localized in the cytoplasm in HEK293 cells (Fig. 1B). In summary, the interaction between Nsp12 and GAL3 was identified and confirmed using yeast 2-hybrid assay, Co-IP, and co-localization assays.

We then investigated the effect of PRRSV infection on endogenous GAL3 expression. Endogenous GAL3 expression was analyzed in MARC-145 cells at different time points after the infection of PRRSV at MOI of 0.1. As shown in Fig. 1C, GAL3 expression was upregulated at 24 h post-infection (hpi) and substantially downregulated in response to PRRSV infection at 36- and 48-hpi compared to its expression level at 24 hpi. We then analyzed the kinetics of *GAL3* mRNA expression in mock- or PRRSV-infected PAMs. Relative expression levels were analyzed using the ΔΔCt method (Bookout et al., 2006) and glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an endogenous control. All the relative *GAL3* levels were quantified as a fold change over that in mock-infected cells at 6 hpi. We observed that the relative expression of porcine *GAL3* was rapidly upregulated at 6 hpi, followed by a subsequent downregulation as a function of PRRSV-infection time (Fig. 1D), indicating that the expression endogenous *GAL3* was negatively correlated with PRRSV replication.

To determine the effect of GAL3 on PRRSV replication, PRRSV infection was initiated at 36 hpt, and the viral titer was assessed in MARC-145 cells. In GAL3-c-Myc-pCAGGS-transfected cells, viral titer was significantly lower than that in the corresponding control at different time points (Fig. 2A), indicating that GAL3 exhibited a strong antiviral activity against PRRSV replication. To confirm the inhibitory effect of GAL3 on PRRSV activity, we performed immunofluorescence assays using a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against the PRRSV nucleocapsid (N) protein. Consistent with viral-titer data, the N protein levels were decreased (Fig. 2B). To further corroborate our findings, the co-expression of transfected GAL3 and the N protein was confirmed by WB, with results indicating that GAL3 overexpression strongly reduced the accumulation of the PRRSV N protein (Fig. 2C). We then assessed the antiviral activity of GAL3 against other PRRSV strains, including an attenuated type 2 vaccine virus (vHuN4-F112; derived from vHuN4; GenBank accession No. EF635006), a classic type 2 strain (vAPRRS; GenBank accession No. GQ330474), and a classic type 1 strain (vSHE; GenBank accession No.

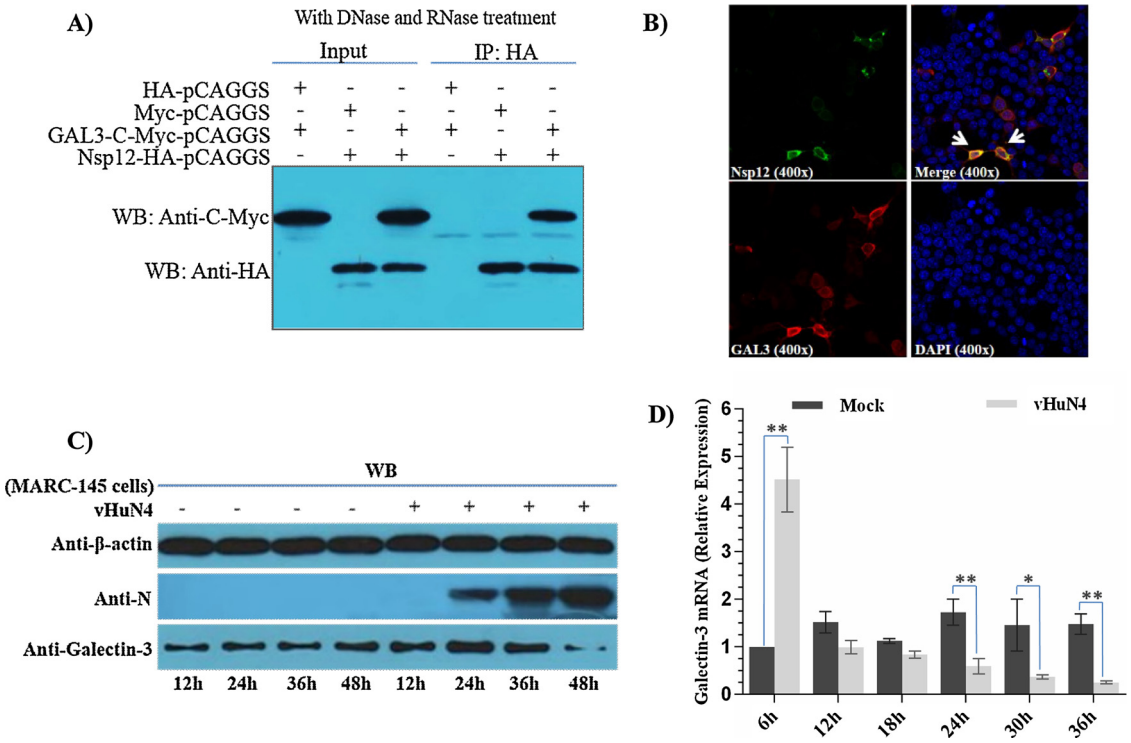

Fig. 1. Interaction between GAL3 and PRRSV Nsp12 and the time course of endogenous GAL3 expression. (A) GAL3 interaction with Nsp12 according to Co-IP results from in HEK293 cells. (B) Co-localization of Nsp12 with GAL3 in HEK293 cells. Cells were fixed and double stained at 24 hpt with a rabbit anti-HA antibody and a mouse anti-Myc antibody, followed by FITC-conjugated anti-rabbit IgG (green) and Alexa Fluor-conjugated anti-mouse IgG (red). Cell nuclei were counterstained with DAPI (1 μg/mL). Time course of endogenous GAL3 expression following infection of (C) MARC-145 cells and (D) PAMs with PRRSV vHuN4 at an MOI of 0.1. Cells were collected at the indicated times, and WB or quantitative reverse transcription PCR was performed to detect GAL3 levels in PRRSV-infected cells relative to mock cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).