

Intracellularly expressed nanobodies against non-structural protein 4 of porcine reproductive and respiratory syndrome virus inhibit virus replication

Hongliang Liu · Chao Liang · Hong Duan ·
Xiaobin Zhang · Xiangpeng Wang ·
Shuqi Xiao · En-Min Zhou 

Received: 28 January 2016 / Accepted: 17 March 2016 / Published online: 24 March 2016
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Abstract

Objective To isolate specific nanobodies to porcine reproductive and respiratory syndrome virus (PRRSV) non-structural protein 4 (Nsp4) and investigate their potential antiviral activities.

Results Three PRRSV Nsp4-specific nanobodies were isolated from a phage display library of the variable domains of camelid heavy chain-only antibodies. Nanobody genes were introduced into MARC-145 cells using lentivirus vectors to establish cell lines stably expressing nanobodies. These intracellularly expressed nanobodies were tested for interaction with PRRSV-encoded Nsp4 within PRRSV-infected MARC-145 cells. Nb41 and Nb43 intrabodies each

potently inhibited PRRSV replication, protected MARC-145 cells from PRRSV-induced cytopathic effect and fully blocked PRRSV replication at an MOI of 0.001 or lower.

Conclusion Intracellularly expressed Nb41 and Nb43 potently suppressed PRRSV replication in MARC-145 cells. Nanobodies hold great potential for development as novel antiviral treatments for PRRSV infection.

Keywords Intrabody · Lentivirus · Nanobody · Non-structural protein 4 · Porcine reproductive and respiratory syndrome virus · Single-domain antibody

Hongliang Liu and Chao Liang contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-016-2086-3) contains supplementary material, which is available to authorized users.

H. Liu · C. Liang · H. Duan · X. Zhang ·
X. Wang · S. Xiao · E.-M. Zhou (✉)
Department of Preventive Veterinary Medicine, College
of Veterinary Medicine, Northwest A&F University,
Yangling 712100, Shaanxi, China
e-mail: liuhongliang55@126.com

C. Liang
e-mail: chaonvlc@163.com

H. Duan
e-mail: duanhong910924@sina.com

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is currently one of the most economically

X. Zhang
e-mail: xiaobin1109@163.com

X. Wang
e-mail: wangxiangpeng2003@126.com

S. Xiao
e-mail: xiaoshuqi@nwsuaf.edu.cn

E.-M. Zhou
e-mail: zhouem@nwsuaf.edu.cn

important viral pathogens threatening the swine industry worldwide (Holtkamp et al. 2013). Current vaccination strategies cannot effectively control PRRSV transmission and no antiviral drugs against PRRSV are commercially available (Murtaugh and Genzow 2011). Therefore, the development of novel anti-PRRSV strategies has become an important area of research.

PRRSV is an enveloped, single-stranded, positive-sense RNA virus that belongs to the order *Nidovirales*, family *Arteriviridae*. The genome is approx. 15 kb in length and contains at least 11 ORFs (Li et al. 2015). ORF1a and ORF1b encode two large replicase polyprotein precursors, 1a and 1ab, which undergo rapid autocatalytic release of non-structural proteins 1 (Nsp1) and 2 (Nsp2), respectively. The remaining polyprotein precursors (Nsp3-8 and Nsp3-12) are cleaved into mature non-structural proteins by non-structural protein 4 (Nsp4), a 3C-like serine protease (3CLSP) (Li et al. 2015; Tian et al. 2009; Xu et al. 2010). In addition, Nsp4 impairs the initial host antiviral responses by interfering with the NF- κ B signaling pathway through the cleavage of NF- κ B essential modulator, leading to the down regulation of IFN- β expression, (Huang et al. 2014). The important roles of Nsp4 in the PRRSV life cycle make it an attractive target for the development of anti-PRRSV agents.

Antibody fragments expressed intracellularly, termed intrabodies, can be used as antiviral agents by inhibiting key viral protein functions inside infected host cells (Lo et al. 2008). This strategy has certain advantages over the use of phage display-selected peptides or RNA interference, because intrabodies have much higher specificity against target proteins and far longer half-lives. Conventional intrabodies are based mainly on engineered single-chain antibody variable domain fragments (scFvs); however, the cytoplasmic expression of functional scFv is generally limited due to misfolding, insolubility and aggregation (da Silva et al. 2004). The use of camel single-domain antibodies (sdAbs) has produced major improvements in results. In addition to conventional antibodies, camelids also produce antibodies without a light chain, so-called heavy chain-only antibodies (HCAbs). The antigen-binding properties of HCAbs are provided by the variable region of HCAbs (VHH), which has been

termed as camel sdAb, or nanobody (Conrath et al. 2003). Nanobodies are ideal candidates for intracellular expression because their intrinsic stabilities are sufficient for proper intracellular folding and function (Rothbauer et al. 2006). Several camel single-domain intrabodies targeting viral proteins have been reported and exhibit antiviral activity (Boons et al. 2014). Previously, we generated a nanobody (designated Nb6) specific against PRRSV Nsp9 and when Nb6 was expressed in the cytoplasm of MARC-145 cells using the eukaryotic expression vector pEGFP-N1 it strongly inhibited the replication of PRRSV in the cells (Liu et al. 2015). In the present study, PRRSV Nsp4-specific nanobodies were isolated via phage display followed by delivery of the nanobody genes into MARC-145 cells using lentivirus vectors. Results show that intracellularly expressed Nb41 and Nb43 potently suppressed PRRSV replication in MARC-145 cells.

Materials and methods

Cells and virus

HEK293T and MARC-145 cells were cultured in DMEM (Gibco, USA) supplemented with 10 % (v/v) fetal bovine serum (FBS) at 37 °C in 5 % CO₂. HP-PRRSV/SD16 (GenBank ID: JX087437), was propagated and titrated in MARC-145 cells grown in DMEM supplemented with 3 % (v/v) FBS.

Construction of plasmids

The cDNA sequence encoding PRRSV Nsp4 was amplified by PCR from the infectious PRRSV cDNA clone pBAC-SD16^{FL} (Wang et al. 2013) using the primer pair Nsp4-F/Nsp4-R. The resulting fragment was then inserted into the pET-28a vector at the *Nco*I and *Xho*I restriction sites to generate the pET-28a-Nsp4 plasmid. The nanobody gene sequence (GenBank ID: KU555411, KU555412, KU555413) was amplified using the Nb-F/Nb-R primer pair, and the gene for EGFP was amplified using the EGFP-F/EGFP-R primer pair. The two fragments were then mixed together in an equal molar ratio to serve as template for a second round of PCR using primer pair

Nb-F/EGFP-R. The resulting fragment was inserted into the pTRIP-CMV-Puro vector (Du and Tikoo 2010) at the *Xba*I and *Bam*HI restriction sites to construct the pTRIP-CMV-Nbx^{EGFP}-Puro plasmid. The sequences of primers used in the study are listed in Supplementary Table 1.

Expression and purification of PRRSV Nsp4 recombinant protein

Plasmid pET-28a-Nsp4 was transformed into *E. coli* (DE3). Expression of recombinant protein in the transformed cells was induced with 1 mM IPTG for 16 h at 25 °C. Bacterial pellets were harvested and soluble N-terminal His-tagged recombinant fusion protein, Nsp4-NHis, was purified as previously described (Liu et al. 2015).

Bactrian camel immunization and library construction

An adult male bactrian camel was immunized subcutaneously with the purified Nsp4-NHis recombinant protein at days 1, 14, 28, 42, 56 and 70. The antibody response was evaluated by titration of serum samples in an enzyme-linked immunosorbent assay (ELISA) as previously described (Liu et al. 2015). Peripheral blood mononuclear cells (PBMCs) were isolated from a blood sample taken 4 days after the last immunization (d 70). A camel VHH library was then constructed as previously described (Liu et al. 2015). All animal experiments were approved by the Northwest A&F University Animal Ethics Committee.

Selection and identification of Nsp4-specific nanobodies

Biopanning was performed as described previously (Vincke et al. 2012). The titers of phage particles in inputs and outputs at all steps were estimated to monitor the enrichment of specific phage particles. After three rounds of panning, 48 clones were randomly picked and the DNA coding for the VHH of each clone was sequenced. Clones were then grouped according to their complementarity determining region (CDR) sequences. The clones were treated with 1 mM IPTG to induce expression of soluble periplasmic nanobody fragments. Periplasmic

extracts were tested for the presence of Nsp4-specific nanobodies using indirect ELISA (Liu et al. 2015).

Lentivirus production and establishment of stable cell lines expressing nanobodies

To produce pseudotyped lentivirus particles, HEK293T cells in 60-mm dishes were co-transfected with three plasmids, psPAX2 (1.2 µg), pMD2.G (1.2 µg) and pTRIP-CMV-Nbx^{EGFP}-Puro (2.4 µg), using X-tremeGENE HP DNA Transfection Reagent (Roche Life Science, USA) according to the manufacturer's instructions. At 72 h post-transfection, virus-containing supernatants were harvested, centrifuged to remove residual cells and were added to MARC-145 cells. Hexadimethrine bromide was added at 8 µg/ml. At 36 h post-transduction, selection of nanobody-expressing cells was performed by addition of puromycin at 5 µg/ml. Surviving cells were monitored using fluorescence microscopy and western blot analysis was used to confirm expression of nanobody-EGFP fusion proteins. MARC-145 cell lines stably expressing nanobody-EGFP fusion proteins were designated MARC-145-Nbx^{EGFP}. Cell growth curves were plotted to visualize proliferation profiles of the cell lines.

Reverse transcription-qPCR (RT-qPCR)

For RT-qPCR assays, total cellular RNA was isolated using RNAiso Plus reagent and reverse transcribed using a Primescript RT reagent Kit (TaKaRa) according to the manufacturers' instructions. The RT-qPCR reaction was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using a FastStart Universal SYBR Green Master (Rox) (Roche Life Science, USA) and ORF7-F/ORF7-R primer pair (Supplementary Table 1). β-actin mRNA served as an internal reference.

Co-immunoprecipitation

PRRSV-infected MARC-145-Nbx^{EGFP} cell lines were homogenized in NP40 lysis buffer. After a centrifugation step, the supernatant was incubated with GFP-Trap[®]_A beads (Chromotek, Germany) at 4 °C for 2 h, then the beads were washed in 1 ml PBS three times. After the last wash step, the beads were resuspended with SDS sample buffer and boiled for 10 min at 95 °C. Immunoprecipitated proteins were

then analyzed by western blot with mouse anti-GFP monoclonal antibody (Tiangen, China) and mouse anti-Nsp4 polyclonal antibody (produced in our laboratory).

Statistical analysis

All experiments were performed as at least three independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA). A p value < 0.05 was considered to be statistically significant.

Results and discussion

Construction of a phage display VHH library from an Nsp4-immunized camel

For construction of a camel VHH library, soluble Nsp4-NHis recombinant protein was expressed in *E. coli* and purified to near homogeneity by two-step chromatography using a Ni-NTA column followed by a Superdex 75 gel filtration column (Supplementary Fig. 1). An adult male bactrian camel was repeatedly immunized with the purified protein over a period of 10 weeks. Serial dilutions of camel sera showed high titers of anti-Nsp4 antibody after the last immunization, as compared to the titer in un-immunized camel serum as determined by indirect ELISA (Supplementary Fig. 2). A phage display VHH library, containing approx. 2×10^8 individual transformants, was built from PBMCs of the immunized camel. After colony PCR analysis of 50 randomly selected clones, 98 % (49) exhibited inserts of the expected size for the VHH gene. DNA inserts of the 49 positive clones were sequenced and all represented distinct VHH sequences, highlighting the diversity and high quality of the initial library.

Isolation and characterization of Nsp4-specific nanobodies

To select nanobodies that specifically bound to PRRSV Nsp4, the phage display library was panned to select for phage that specifically bound to immobilized recombinant protein Nsp4-NHis. After the third round of panning, a strong enrichment of phage particles carrying Nsp4-specific VHHs was observed (Supplementary Table 2) and 48 clones were

randomly selected for sequencing. These clones represented four distinct individual VHHs, namely Nb41, Nb42, Nb43 and Nb44, that occurred 6, 39, 2 and 1 times, respectively, among the 48 selected clones. Binding capacities and specificities of the nanobodies were determined using indirect ELISA. Nb53, derived from an un-immunized camel VHH library, was used as a negative control (Fig. 1). With the exception of Nb44, all of the selected nanobodies reacted with Nsp4-NHis, but not the negative control, Nsp9-CHis. These results eliminate the possibility that the nanobodies may recognize the $6 \times$ His region.

The amino acid sequences of the three Nsp4-specific nanobodies were aligned against the human clan III VH sequence (Supplementary Fig. 3). This alignment clearly shows that Nb41 and Nb42 were derived from VHH germline genes, as they all contain the hallmark amino acid substitutions in framework 2

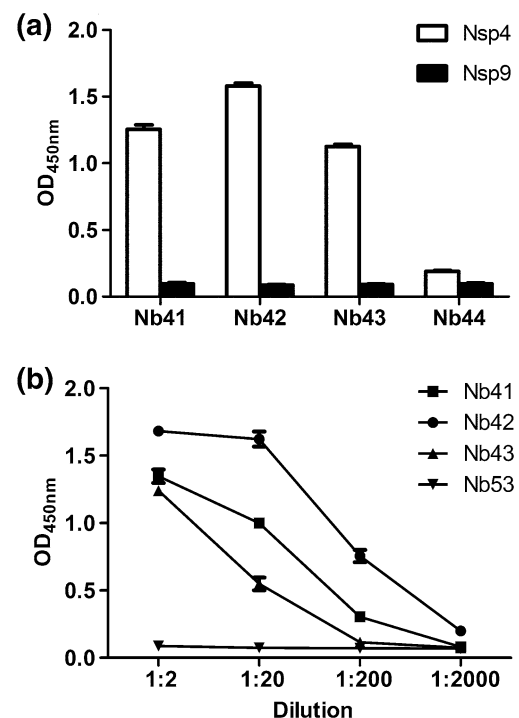


Fig. 1 Binding capacity and specificity of nanobodies against Nsp4. **a** The four unique nanobody fragments extracted from IPTG-induced *E. coli* TG1 colonies were checked for their binding capacities and specificities for immobilized Nsp4 using indirect ELISA with Nsp9-CHis protein as a control. **b** Titration of the nanobodies with Nb53 serving as anti-Nsp4 nanobody negative control. Assays were performed in triplicate and data are presented as mean \pm SD

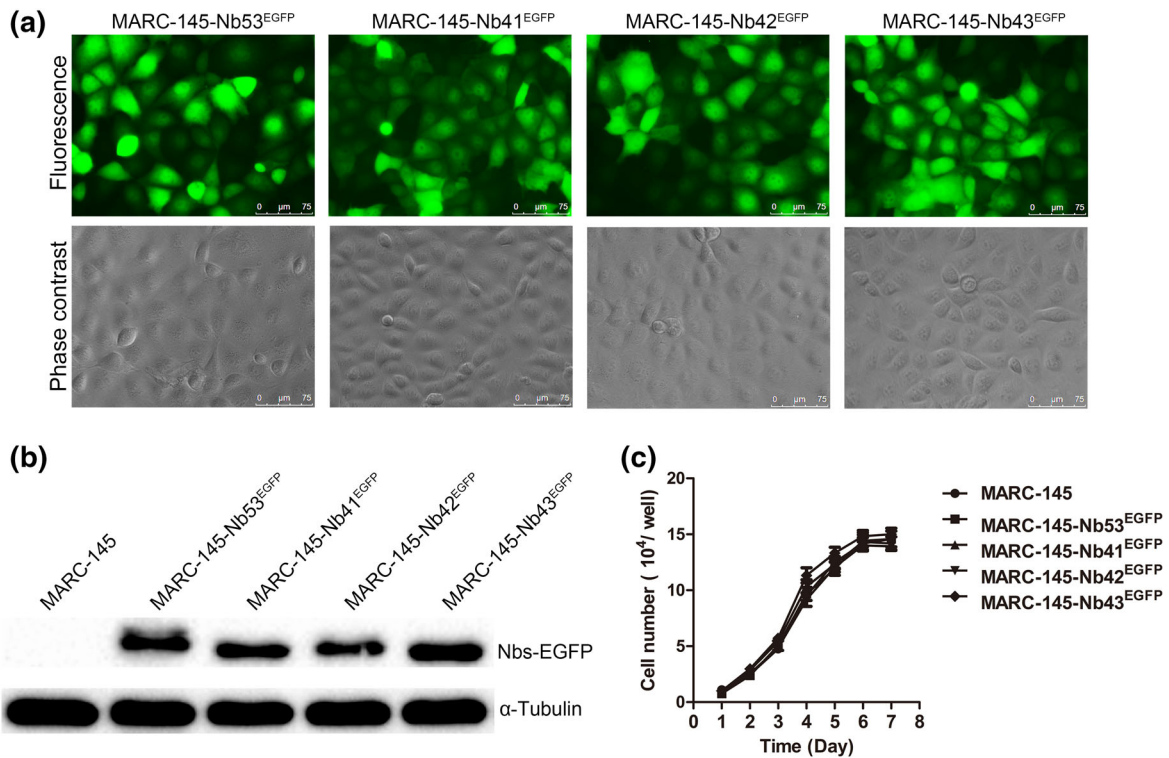


Fig. 2 MARC-145 cell lines stably expressing nanobody-EGFP fusion proteins. **a** Cell lines were imaged using fluorescent microscopy. **b** Expression of nanobody-EGFP fusion proteins was detected by western blot using the anti-GFP monoclonal antibody. **c** Cell growth curves were

determined for cells seeded in 96-well plates (5×10^3 cells/well). On each day of cultivation, half of the culture medium was replaced with fresh medium and cells were trypsinized for cell counting. Assays were performed in triplicate and data are expressed as mean \pm SD

[V37F, G44E, L45R, W47G, using the Kabat numbering system (Kabat et al. 1991)]. These hydrophilic residues have been reported to be important for the solubility and stability of nanobodies (Conrath et al. 2003). Interestingly, Nb43 shows high sequence homology with human clan III VH sequences. This type of VH Ag-binding single-domain Ab fragment has been repeatedly isolated previously in camel VHH libraries. Such Ab fragments, although they lack the hallmark characteristic VHHFR2 residues, still remain highly soluble, stable and functional (Deschacht et al. 2010).

Generation of stable MARC-145 cell lines expressing nanobodies

To generate MARC-145 cell lines stably expressing nanobodies intracellularly, lentivirus TRIP-CMV-

Nbx^{EGFP}-Puro was created and MARC-145 cells were transduced with the pseudotyped lentiviral particles. After screening with puromycin, surviving cells were imaged using fluorescent microscopy (Fig. 2a). Western blot analysis confirmed expression of nanobody-EGFP fusion proteins (Fig. 2b). Bands of the expected size (40 kDa) were detected in MARC-145-Nb53^{EGFP}, MARC-145-Nb41^{EGFP}, MARC-145-Nb42^{EGFP} and MARC-145-Nb43^{EGFP} cells after incubation with mouse anti-GFP mAb, but not in wild type MARC-145 cells. Analysis of cell proliferation of the cell lines showed that nanobody-EGFP fusion proteins exhibit neither cytotoxicity nor any negative effect on the growth of MARC-145 cells (Fig. 2c).

Lentiviral vectors are useful tools for gene delivery because of their ability to transduce a wide variety of both dividing and non-dividing cells and achieve stable and long-term transgene expression, emphasizing their utility for gene

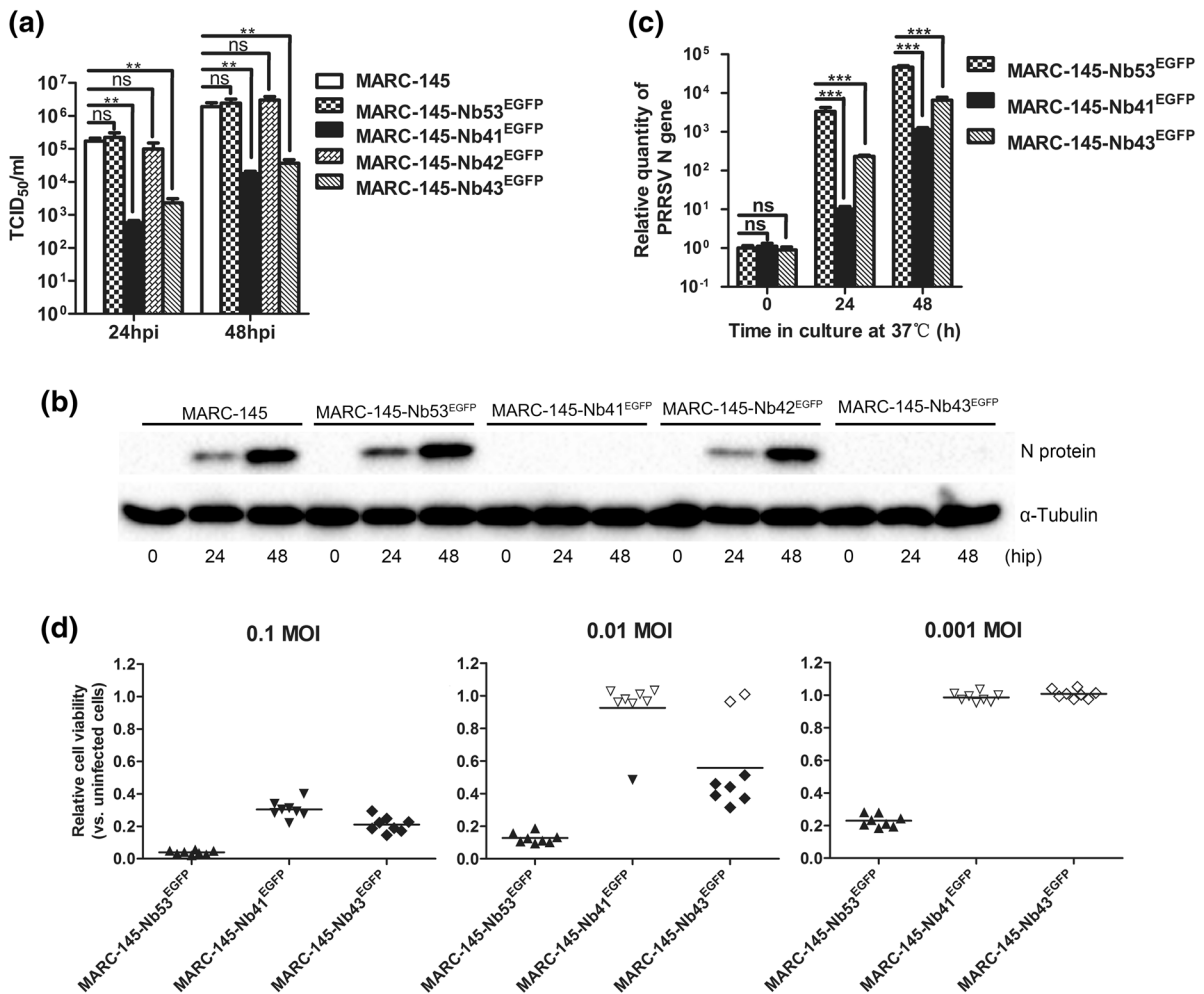


Fig. 3 Antiviral effects of intracellularly expressed nanobodies. **a** Inhibition of progeny virus production by intrabodies Nb41 and Nb43. Cell lines were infected with SD16 (0.1 MOI). At 24 and 48 hpi, the titers of progeny virus were measured by TCID₅₀ assay and data are expressed as mean ± SD of three independent experiments. Significant differences as compared with MARC-145 cells are denoted by (***p* < 0.01). **b** Inhibition of PRRSV N protein expression by intrabodies Nb41 and Nb43. Cells were harvested at 24 and 48 hpi, N protein was detected by western blot using a PRRSV N protein-specific monoclonal antibody (6D10). **c** Inhibition of PRRSV N mRNA levels by intrabodies Nb41 and Nb43. Cells were incubated with PRRSV SD16 (1 MOI) at 4 °C for 1 h then washed with ice-cold PBS and transferred to 37 °C for culture. After culturing for 0, 24 and

48 h, cells were harvested and relative PRRSV N mRNA levels were detected using RT-qPCR with PRRSV N gene-specific primers. β-Actin mRNA served as internal reference. Significant differences compared with MARC-145-Nb53^{EGFP} cells are denoted by (***p* < 0.001). **d** Intrabodies Nb41 and Nb43 suppressed PRRSV-induced CPE and fully blocked PRRSV replication at low MOI. Cells were infected with SD16 at different MOIs. At 5 dpi, cell viability was assessed using the WST-8 method; results of individual wells are expressed as relative cell viability of infected cells versus uninfected cells. Culture supernatants were tested for the presence of infectious PRRSV; solid symbols represent infectious PRRSV positive supernatants, while open symbols represent infectious PRRSV negative supernatants

therapy (Escors and Breckpot 2010). Results presented here confirm that lentiviral vectors are efficient delivery systems for introduction of nanobody genes into MARC-145 cells to achieve intrabody expression.

Intracellularly expressed Nb41 and Nb43 suppress PRRSV replication

To determine the potential inhibitory effect of intracellularly expressed nanobodies on PRRSV

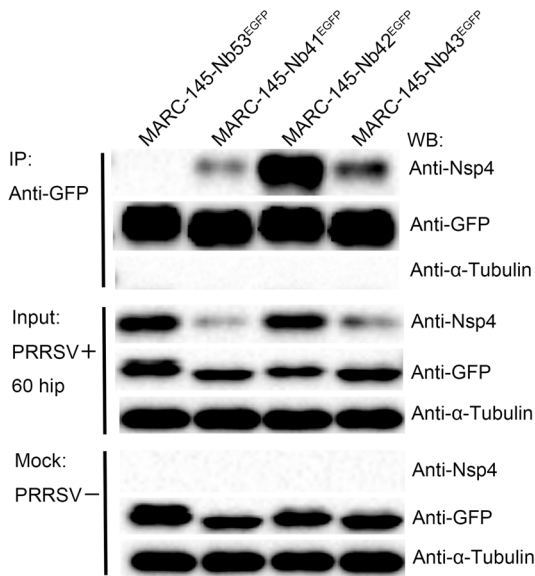


Fig. 4 Interaction of intracellularly expressed nanobodies with PRRSV-encoded Nsp4 in MARC-145 cells. Cells were infected with PRRSV at an MOI of 1. At 60 hpi, immunoprecipitations of cell lysates were performed using GFP-Trap_A beads. Immunoprecipitated proteins were then analyzed by western blotting using mouse anti-GFP monoclonal antibody and mouse anti-Nsp4 polyclonal antibody

multiplication, MARC-145 cells and MARC-145 cell lines stably expressing nanobodies were infected with PRRSV strain SD16 (0.1 MOI). At 24 h post-infection (hpi) (Fig. 3a), intrabodies Nb41 and Nb43 inhibited infectious virus release by about 99.7 and 99 %, respectively, as compared with virus release in MARC-145-Nb53^{EGFP} cells. At 48 hpi, the Nb41 and Nb43 inhibition rates were 99.2 and 98.5 %, respectively. The control intrabody, Nb53, and the Nsp4-specific intrabody, Nb42, had no significant influence on PRRSV multiplication, as compared with wild type MARC-145 cells. These inhibition results were confirmed by PRRSV N protein expression patterns in the cell lines (Fig. 3b). Next, PRRSV attachment to cells and replication in cells were tested by RT-qPCR. As shown in Fig. 3c, although there was no significant difference in the relative PRRSV N mRNA levels among the cell lines after virus attachment at 4 °C, PRRSV N mRNA levels in MARC-145 cells expressing Nb41 or Nb43 were strongly decreased.

To further evaluate the antiviral capacity of intrabodies Nb41 and Nb43, cell lines were infected with SD16 at different MOIs. At 5 days post-infection (dpi), culture supernatants were tested for the presence of infectious PRRSV and relative cell survival rate was assessed by the WST-8 cell proliferation method (Fig. 3d). Intrabodies Nb41 and Nb43 protected MARC-145 cells from any virus-induced cytopathic effect (CPE) and fully blocked PRRSV replication at an MOI of 0.001 or lower. Similarly, the anti-PRRSV effect of the Nsp9-specific intrabody, Nb6, had been reported in our previous study (Liu et al. 2015). Because Nsp4 is the main PRRSV proteinase responsible for most viral non-structural protein processing, its critical role in the virus life cycle makes it an ideal antiviral target candidate. Furthermore, the results reported in this study indicate the importance of Nsp4 on PRRSV replication. The success of combination antiretroviral therapies for HIV infection has been reported by targeting at least two distinct molecular, the reverse transcriptase and protease (Maenza and Flexner 1998) indicating that it is ideal for future development of a combination drug therapy by targeting Nsp4 (Liu et al. 2015) and Nsp9 as demonstrated in this report to combat this important swine disease.

Intracellularly expressed nanobodies interact with PRRSV-encoded Nsp4

To confirm the interaction of intracellularly expressed nanobodies with PRRSV-encoded Nsp4 in PRRSV-infected MARC-145 cells, co-immunoprecipitation assays were carried out on lysates of PRRSV-infected MARC-145 cell lines stably expressing nanobodies. As shown in Fig. 4, all of the three nanobodies could interact with PRRSV-encoded Nsp4 in PRRSV infected MARC-145 cells. The lower expression level of Nsp4 in MARC-145-Nb41^{EGFP} and MARC-145-Nb43^{EGFP} cell was due to the inhibition of PRRSV replication by intrabody Nb41 and Nb43. Above results suggest that intrabody Nb42 recognize a non-functional epitope of Nsp4, by contrast, intrabody Nb41 and Nb43 inhibit PRRSV replication by recognizing functional epitopes of Nsp4. Epitope mapping of the three Nsp4-specific nanobodies is still in progress and should hold great significance for the

rational design of antiviral agents, as well as for our future understanding of the biological characteristics of Nsp4.

Conclusion

PRRSV Nsp4-specific nanobodies were isolated from a camel VHH library for the first time. Nb41 and Nb43 potently suppressed PRRSV replication upon expression in MARC-145 cells, suggesting that they may be promising candidates as novel therapeutic agents against PRRSV infection pending development of appropriate delivery and application systems. Moreover, these nanobodies may also serve as useful tools to further our understanding of the biological functions of Nsp4 in the PRRSV life cycle.

Acknowledgments This study was funded by the National Natural Science Foundation of China (31430084).

Supporting information Supplementary Table 1—Primers used in this study.

Supplementary Table 2—Enrichment of Nsp4-specific phages during subsequent rounds of panning.

Supplementary Figure 1—Expression and purification of the Nsp4-NHIS recombinant protein.

Supplementary Figure 2—Camel immune response raised against Nsp4.

Supplementary Figure 3—Amino acid sequence alignment of isolated nanobodies with human VH (hVH).

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