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RNAi-based inhibition of porcine reproductive and respiratory syndrome virus replication in transgenic pigs

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating viral disease causing heavy losses to the swine industry worldwide. Many studies have shown that transient delivery of small interfering RNA (siRNA) or adenovirus-mediated RNA interfere (RNAi) could potentially inhibit porcine reproductive and respiratory syndrome virus (PRRSV) replication *in vivo* and *in vitro*. Here, we applied RNAi to produce transgenic (TG) pigs that constitutively expressed PRRSV-specific siRNA derived from small hairpin RNA (shRNA). First, we evaluated siRNA expression in the founding and F1 generation pigs and confirmed stable transmission. Then, we detected the expression of IFN-β and protein kinase R (PKR) and found no difference among TG, non-transgenic (NTG), and wild-type pigs. Lastly, the F1 generation pigs, including TG and NTG piglets, were challenged with $3 \times 10^{4.5}$ $TCID_{50}$ of JXA1, a highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV). Our results showed that the *in vivo* siRNA expression substantially reduced the serum HP-PRRSV titers and increased survival time by 3 days when TG pigs were compared with the NTG controls. These data suggested that RNAi-based genetic modification might be used to breed viral-resistant livestock with stable siRNA expression with no complications of siRNA toxicity.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious disease and one of the most devastating to the swine industry worldwide. PRRS is characterized by severe reproductive failure in gilts and respiratory illness in pigs of all ages. The etiological agent PRRS virus (PRRSV) is a member of the family Arteriviridae that contains a linear, positive-sense, single-stranded RNA genome and transcripts using a unique mechanism involving discontinuous subgenomic synthesis (Meulenberg et al., 1993). And an unprecedented "high fever" disease caused by HP-PRRSV broke out in China in 2006, which spread more than 10 provinces and brought about 4,000,000 fatal cases (Tian et al., 2007). Currently, the principal method to control and prevent PRRSV infection is vaccination. However, the vaccines are unable to provide potent and lasting disease control because of the immune evasion strategies of PRRSV and the heterogeneity of its genome (Thanawongnuwech and Suradhat, 2010).

RNA interference (RNAi) is an RNA-guided sequence-specific process in which small interfering RNA (siRNA) induces post-transcriptional gene silencing. RNA silencing was initially recognized as an anti-viral mechanism to protect host organisms from RNA viral infections (Waterhouse et al., 2001). Many studies have confirmed antiviral immunity mediated by virus-derived small interfering RNAs (viRNAs) in plants and invertebrates, although viRNAs have not been identified in mammals which have evolved the interferon (IFN) pathway instead (Ding and Voinnet, 2007). It has been demonstrated that siRNA can elicit specific intracellular antiviral resistance and may, therefore, be a valuable therapeutic tool (Gitlin et al., 2002). Hence, RNAi has great potential for the inhibition of RNA viral replication *in vitro* and *in vivo*. Since the first reported case of RNAi therapy against human pathogen respiratory syncytial virus (RSV) (Bitko and Barik, 2001), many other

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types of human viruses have been successfully targeted, including human immunodeficiency virus type 1 (Coburn and Cullen, 2002), hepatitis C virus (Kronke et al., 2004), hepatitis B virus (McCaffrey et al., 2003), severe acute respiratory syndrome (He et al., 2003), and influenza A virus (Ge et al., 2003). Subsequently, many viruses infecting large farm animals have also been chosen as therapy targets, such as foot and mouth disease virus (Chen et al., 2006), porcine circovirus virus type 2 (Feng et al., 2008), porcine endogenous retrovirus (Ramsoondar et al., 2009), and PRRSV (Li et al., 2009) (Guo et al., 2013).

A recent report has declared that siRNA transgenesis could confer viral infection resistance to pseudorabies virus (PRV) on mice (Daniel-Carlier et al., 2013). Although it has been shown that transient delivery of siRNA by targeting viral genes protects susceptible cell lines (He et al., 2007), stable transgenic cell line presented stable inhibition to virus (Zhou et al., 2012), and recombinant adenovirus-mediated RNAi guards animals against viral challenges (Li et al., 2009), it remains unclear whether transgenesis of siRNA in pigs can confer permanent resistance against PRRSV infection. Previously, we designed siRNAs to target the open reading frame (ORF) 1b and 6 regions of PRRSV and showed that siRNA-1B372 targeting ORF1b had the strongest virus-inhibiting effect in MARC-145 cells. Importantly, the viral yield in siRNA-1B372-transfected cells was 600-fold lower than that in control cells (Bao et al., 2012). ORF1b encodes non-structural proteins (NSP), including NSP 9–12, which plays a key role in the initiation of viral replication (Music and Gagnon, 2010). Based on our previous *in vitro* data, our aim in the present study was to produce transgenic animals perpetually expressing siRNA-1B372 and then challenge these animals with PRRSV. Here, we report that we generated transgenic pigs with stable siRNA expression over two generations, and the reduction of PRRSV viremia was observed in them.

2. Materials and methods

2.1. Plasmid construction

Based on our previous findings (Bao et al., 2012), we selected siRNA-1B372 as an RNAi trigger, targeting sequence 5′-GGTGTACTTCCGTTCATTA-3′ (9323–9341 nt) of PRRSV RNA genome. Double-stranded (ds) oligonucleotides encoding siRNA-1B372 were annealed and inserted into vectors and then cloned into a *Bam*HI/*Hind*III-linearized pGenesil-1 plasmids (Wuhan Genesil Biotechnology, Hubei, China) containing the human U6 (hU6) promoter to generate pshRNA-1B372 (Fig. 2a). Further details of vector construction are described in our previous study (Bao et al., 2012).

2.2. Generation of transgenic pigs

Fetal pig fibroblast cell lines (from Landrace pigs) were established and cultured as described previously (Zhang et al., 2006). The fibroblast cells were transfected with linearized shRNA-expressing constructs, and then cultured on a 10-cm plate. After 24 h, the cells were transferred to six 10-cm plates with selective medium that contained G418 (400 μg/ml, Promega). After selection with G418 for a week, the antibiotic-resistant colonies were selected and pooled in 24-well plates. Forty-eight hours later, half of the cells in each well were subjected to PCR using primers 1B372-F (5′-CTGTTCCACATACACTTCATTCT-3′) and 1B372-R (5′-CACAGATGCGTAAGGAGAAA-3′) to identify the transgenic positive clones. The other half of the cells was frozen for later use in somatic cell nuclear transfer (SCNT). Approximately, 400 embryos were transferred into each surrogate sows and five sows received embryo implantation. Cloned pigs were delivered by natural birth after approximate 106 days, and F1 is generated by fertilizing TG sows with semen of wild-type pigs.

TG founder pigs and offspring were identified by polymerase chain reaction (PCR) analysis of genomic DNA derived from ear biopsies. Two sets of primers were designed to cover the integrity of the 5′-region (UPPER1F: 5′-CAGCGGTAAGATCCTTGAG-3′; UPPER1R: 5′-ACTCCCCGTCGTGTAGATAAC-3′) and the 3′-region (LOWER1F: 5′-ATTCCCCCAGTGGAAAGAC-3′; LOWER1R: 5′-TGCGGCATCAGAGCAGATT-3′). TG founder pigs were confirmed by Southern blot analysis. The plasmid vector was served as positive control. Copy number standards (the number of inserted vector DNA per diploid genomic equivalent) are indicated as 1c, 3c and 5c, and the corresponding amount of plasmid loaded in each well was calculated according to the equation:

$$f(c) = \frac{10 \times l \times c}{2 \times L}$$

l, the length of the plasmid; c, the copy number standard; $2 \times L$ means diploid genome. Proper amount of plasmid and ear DNA samples (10 μg) were digested with *Pvu*II and electrophoresed through a 1% agarose gel followed by transfer to positively charged nylon membranes (Roche Applied Science, Mannheim, Germany). The membrane was hybridized with adigoxigenin (DIG)-labeled probes (761 bp) generated by PCR using the following set of primers (PROBE2F: 5′-ACTGGGCACAACAGACAATC-3′ and PROBE2R: 5′-CGATCCCCTCAGAAGAACTC-3′) with the pGenesil-1b273 plasmid as the template (Roche DIG-PCR DNA Labeling Kit). Hybridization signals were detected using the Roche DIG DNA Labeling and Detection Kit according to the manufacturer's instructions.

2.3. Determination of transgene copy number

To determine the transgene copy number, the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to compare the ΔCt value (cycle threshold (Ct); Ct of target minus Ct of control gene) of TG animal samples with unknown transgene copy numbers with the ΔCt value of a known calibrator. A wild-type (WT) pig genomic DNA sample, carrying a single copy of the myostatin (MSTN) gene, was used as a calibrator to determine the transgene copy number in TG founder pigs (MSTN110-F: 5′-TCTGAGACCCGTCAAGACTCCTA-3′, MSTN110-R: 5′-TGTCAAGTTTCAGAGATCGGATTC-3′). The transgene was amplified using 5′-CTGAAG CGGGAAGGGACTGG-3′ and 5′-TACTTTCTCGGCAGGAGCAA-3′ as specific primers.

Quantitative real-time PCR (qRT-PCR) was used to detect the copy number, and all of the reactions were performed in 96-well plates using the Roche LightCycler 480 System (LC 480, Roche, Basel, Switzerland). The amplification was performed in a 20-μL reaction volume containing 1 μl of template DNA (10 ng/μl), 0.3 μl of each primer (10 μM), 10 μl of Power SYBR Green Mix (Applied Biosystems, Foster City, CA, USA), and 8.4 μl of ddH_2O. All reactions were performed using the following cycle conditions: 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s; followed by 95 °C for 5 s, 65 °C for 1 min, and a final cycle at 97 °C to generate a melting curve. A standard set of mixtures, representing 1, 2, 4, 8, 16, and 32 copies of plasmid DNA in 10 ng of WT pig genomic DNA, was used to construct a standard curve to determine the correlation between the ΔCt value (Ct of the pshRNA-1B372 gene minus Ct of MSTN) and the $\log_2 N$ value (N = copy number of the transgene).

2.4. Detection of siRNA by real-time PCR

We used Custom TaqMan Small RNA Assays (Applied Biosystems) to evaluate siRNA expression in TG pigs (Chen et al., 2005). Blood samples of founder were collected. After challenged with PRRSV, all animals' heart, liver, spleen, lung, tonsil and blood were