Classical swine fever virus replicated poorly in cells from MxA transgenic pigs

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Abstract

Background: In addition to their value as livestock, pigs are susceptible to classical swine fever virus (CSFV) and can serve as reservoirs for CSFV, allowing it to develop into an epizootic. CSFV, a pestivirus of the Flaviviridae family, has a single-stranded RNA genome. Recent research has indicated that the human MxA protein inhibits the life cycles of certain RNA viruses, such as members of the Bunyaviridae family, the Flaviviridae family and others.

Results: To produce pigs with antiviral protection against CSFV, transgenic pigs expressing human MxA were generated by nuclear transplantation. Cells from three MxA transgenic piglets were used to investigate in vitro antiviral activity of MxA against CSFV, and the results of in vitro indirect immunofluorescence assays, virus titration and real-time PCR indicated that the MxA transgenic pig has an antiviral capacity against CSFV.

Conclusions: Transgene with human MxA on pigs is feasible. High levels of MxA expression do inhibit CSFV in vitro at early time points post-infection at 60-96dpi.

Keywords: Classical swine fever virus, MxA, Nuclear transplantation, Transgenic pig

Abbreviations: CSFV, Classical swine fever virus; EF1a, Elongation factor 1 alpha; H5N1, Influenza A virus subtype H5N1; MxA, Myxovirus resistance gene A; NLS, Nuclear localization signal; NT, Nuclear transfer; ORF, Open reading frame

Background

Human MxA has been shown to exhibit a wide antiviral capacity against small RNA viruses. This interferon-induced protein is one of the best-studied determinants of innate immunity to viral infection [1]. MxA belongs to the dynamin superfamily of large GTPases and has a C-terminal stretch of basic amino acids that constitutes a nuclear localization signal (NLS). This NLS mediates the nuclear accumulation of MxA, whereas the swine Mx1 protein is not located inside nucleus for lack of NLS. At present, the known MxA-sensitive viruses include members of the bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses and togaviruses, along with Hepatitis B virus [2–7].

Classical swine fever virus (CSFV) is a pestivirus of the Flaviviridae family [8] with an enveloped virion incorporating glycosylated membrane proteins. The CSFV genome has a single strand of positive-sense RNA [9] and a single open reading frame (ORF) that encodes a large protein that is cleaved and modified into several smaller proteins, including N\(^{\text{pro}}\), C, E0, E1, E2, P7, NS2-3, NS4A, NS4B, NS5A and NS5B. CSFV infects pigs and replicates predominantly in myeloid cells, causing classical swine fever (CSF), a severe disease that is characterized by fever, leukopenia and hemorrhage [9–11].

The expression of porcine Mx1 was absent from CSFV-infected pigs because of the Interferon-blocking effects of N\(^{\text{pro}}\) [12, 13]. Moreover, our earlier study concluded that highly expressed MxA inhibits CSFV in vitro [14]. As the inherently wide antiviral capacity of MxA, we sought to generate a transgenic pig model that would express a high level of MxA under the control of the EF1a promoter to protect swine against CSF. In this study, three MxA transgenic pigs were generated by Nuclear transplantation (NT), and we determined the levels at which CSFV replicated in cells from different tissues of these transgenic clone piglets.

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Methods

DNA transfection and the establishment of stable cell lines

The plasmid pGKNteotAloxp2MxA, which expresses human MxA in eukaryotic cells, was constructed in our previous study [14]. Porcine fetal fibroblasts (large white) cultured in Dulbecco’s modified Eagle’s medium (GIBCO) with 20 % fetal bovine serum (PAA) were seeded in a 60-mm dish prior for transfection. Then, the linearized plasmid was transfected into the porcine fetal fibroblasts using Fugene HD Transfection Reagent (Roche). After 14-36 h, the cells were split into 6-well dishes and cultured in selective medium containing 300 ng/mL of G418 antibiotic (Amresco) for 7-12 days. The surviving cell colonies were selected for further analysis.

The generation of transgenic pigs by nuclear transplantation

Positive cell clones were used to construct transgenic pigs by Nuclear transplantation (NT). The details of the method of porcine NT used were described in our earlier work [15]. Briefly, oocytes were washed three times and then matured in maturation medium at 39 °C. In vitro-matured metaphase II oocytes were harvested by the removal of their polar bodies and the associated metaphase plates. An electrical pulse was used to fuse the donor cells and the oocytes. The reconstructed oocytes were electrically activated to initiate cell division and subsequent development. The embryos were cultured for 20 h following activation and were surgically transferred into the oviducts of surrogate pigs.

DNA extraction, RNA extraction, PCR analysis and Integration site analysis

Genomic DNA from different tissues and cells was extracted and purified using a QIAamp DNA Mini Kit (QIAGEN). RNA was extracted with TRIzol reagent (Invitrogen) and purified using an RNeasy column (QIAGEN). The sites of transgene integration were assessed using high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) [16]. Primers specific to the transgenic vectors (pMl1, pMl2 and pMl3) were designed using Vector NTI 10 software (Invitrogen). The detailed method has been previously described [17].

All the primers used in our research and their sequences are listed below:

- MxAq1 GAGAGGAAACTGTAGGGGAG
- MxAq2 GGAAACATCTGTGAAAGCAA
- Neo1 TGAAGTAACTGCAGGACGAG
- Neo2 AATATCAGGGTAGCCACCAG
- β-actin1 GAACCCCAAAGCCAACCGT
- β-actin2 CCTCGTAGATGGGCACCGT
- rFPprimer GGGATGATGGTCTCCTGATCAC
- rRPprimer CATCAAATTTGTTAGGCGACTTTTC
- Prob5B FAM-CCTTGTCCTGCGAATTTCTCACC
- GATAMRA (TagMan)
- Vs1 TCGCCCTTATTCCTCTTTTTT
- Vs2 AGCAGGAGGTTGTCTGTGT
- pMl1 TCGGTGATGACGGTGAAAAC
- pMl2 CGGAGACGGTCACAGCTTGT
- pMl3 AGACAGATGGTACTGAGAG
- LAD1 ACGATGGGACTCAGAGCGGCCGC (G/C/A) N (G/C/A)NNNGAA
- LAD2 ACGATGGGACTCAGAGCGGCCGC (G/C/T) N (G/C/T)NNNGGT
- LAD3 ACGATGGGACTCAGAGCGGCCGC (G/C/A) (G/C/A) N(G/C/A) NNNCCAA
- LAD4 ACGATGGGACTCAGAGCGGCCGC (G/C/T) (G/A/T) N (G/C/T) NNNCGGT
- Vs1 TCGCCCTTATTCCTCTTTTTT
- Vs2 AGCAGGAGGTTGTCTGTGT
- pMl1 TCGGTGATGACGGTGAAAAC
- pMl2 CGGAGACGGTCACAGCTTGT
- pMl3 AGACAGATGGTACTGAGAG
- LAD1 ACGATGGGACTCAGAGCGGCCGC (G/C/A) N (G/C/A)NNNGAA
- LAD2 ACGATGGGACTCAGAGCGGCCGC (G/C/T) N (G/C/T)NNNGGT
- LAD3 ACGATGGGACTCAGAGCGGCCGC (G/C/A) (G/C/A) N(G/C/A) NNNCCAA
- LAD4 ACGATGGGACTCAGAGCGGCCGC (G/C/T) (G/A/T) N (G/C/T) NNNCGGT
- (G/C/A) (G/C/A) N(G/C/A) NNNCCAA
- LAD4 ACGATGGGACTCAGAGCGGCCGC (G/C/T) (G/A/T) N (G/C/T) NNNCGGT
- (G/C/T) (G/A/T) N (G/C/T) NNNCGGT

Viral infections, virus titration, the indirect immunofluorescence assay (IFA) and real-time PCR

These assays were performed according to Xu et al. [18]. CSFV (SM) from the Institute of Veterinary Drug Control, China, was used in this study. Positive anti-CSFV serum was kindly provided by Dr. CC Tu. To determine the viral titer, cells were infected with CSFV for 60 h, and samples of the medium were collected, serially diluted 10-fold from 10⁻¹ to 10⁻⁶ and inoculated onto PK-15 cells. Each dilution was examined for viral proliferation using IFA. The TCID₅₀ values were calculated using the Kärber method. For IFA, cells were infected with CSFV for 72 h, fixed in precooled 80 % acetone, and incubated with the positive anti-CSFV serum. A FITC-conjugated rabbit anti-pig IgG (Sigma) was used as the secondary antibody, and the cells were examined using an ECLIPSE TE2000-V (Nikon). Real-time PCR was performed with an iQ™ Multicolor Real-time PCR Detection System (Bio-Rad) using a QuantiTect™ Probe PCR Kit (QIAGEN). The special primers (rFPprimer, rRPprimer and Prob5B) for the CSFV NS5B gene have been previously described [14].

Western blotting

The cells were washed with PBS and a hypotonic cell lysis buffer containing morpholinepropanesulfonic acid-buffered saline with Triton X-100 (15ul for 10⁶ cells). The cell extract was centrifuged at 12,000 x g to remove the cellular debris. Detailed methods regarding separating the sample by SDS-PAGE, transferring proteins to nitrocellulose membranes, and immunostaining will be described elsewhere. As a control, a monoclonal mouse anti-β-actin antibody (human/mouse, 1:1000, kangchen) was used. The MxA protein was immunostained with a mouse monoclonal anti-MxA (p78) antibody (human/mouse, 1:500 Novus Biologicals).
**Southern blotting**
The sample was digested overnight with EcoRI at 36 °C (1.5ul for 1ug, NEB). Then, the gDNA was electrophoresed for 22 h in a 1 % agarose gel at 13 V and transferred to a Hybond þ nylon membrane (GE Healthcare) for 12 h. A DIG-labeled probe was generated using a PCR DIG Probe Synthesis Kit (Roche) with primers Vs1 and Vs2. Hybridization was performed overnight at 55 ° C using a DIG Easy Hyb Kit (Roche).

**Results**
To select supporting donor cells, several stable MxA-positive clones were constructed, infected with CSFV and analyzed (data not shown). The No. 34 clone (Fig. 1a) was chosen for NT, about 500 SCNT embryos were transferred into 3 surrogate mothers that exhibited natural estrus and three male transgenic piglets were obtained by natural delivery on day 119 post-implantation (Fig. 1b) and one piglet died before birth, the other three live transgenic piglets displayed no obvious weight differences comparing to a normal male piglet born at the same day (Fig. 1c).

The results of PCR detection of the neomycin resistance gene (Neo) from tail DNA and RT-PCR detection of MxA from blood RNA were both positive (Fig. 2a and b). The amplification of template genomic DNA with nested PCR primers yielded amplified product of the predicted size: 163 bp (outer set) and 107 bp (inner set). The PCR-amplified inner set product were sequenced. The sequence analysis revealed that the MxA fragment had randomly inserted into introns on chromosome 16 (NW_003612429:30482-30588). Southern blotting targeting clone piglets tails’ DNA and Western blotting targeting porcine kidney cells were also performed to demonstrate the authenticity of the MxA expression in these transgenic piglets (Fig. 2c and d).

To detect CSFV replication, adherent cells from tails, kidneys and umbilical cords were isolated and cultured. The cells were infected with 100 TCID₅₀ of CSFV for 72 h, and the infected cells were detected using IFA. Only a few MxA-expressing cells exhibited green fluorescence, indicating that most of the cells had been effectively protected by MxA and had resisted viral infection (Figs. 3a-c). To quantify the effect of MxA on viral replication, viral genome copy numbers from porcine tail cells were determined using real-time PCR at 96 hpi. The viral genome copy number per nanogram of total RNA was measured to be $1.05 \times 10^5$ copies/ng of total viral RNA from control cells, whereas the MxA-positive cells contained $1.89 \times 10^3$, $2.31 \times 10^3$, and $2.34 \times 10^3$ copies/ng, corresponding to 55-, 143- and 23-fold reductions, respectively (Fig. 4a). Moreover, the tail cells were infected with 100 TCID₅₀ of CSFV for 60 h, and the virus-containing supernatant was diluted and used to inoculate PK-15 cells to determine the TCID₅₀ values at 60, 72, 84, 96 and 120 h. As shown in Fig. 4b, the MxA protein markedly inhibited infectious virus production at the early time points (60-96hpi) comparing to normal porcine tail cells, however, at later times post-infection (120hpi), the degree of inhibition was reduced (Fig. 4b).

**Discussion**
Transgenic pigs are of great value for commercial applications and could serve as models for human disease [19, 20]. Pigs are closer to humans in complexity,
physiological function and genetics [15, 21]; therefore, we aimed to express human MxA, but not mouse Mx1, in pigs. Somatic cell nuclear transfer is a promising method of producing transgenic animals; however, this cloning strategy has a low success rate (<2 %) for bringing transferred pig embryos to term [22]. It has been well established that unknown mechanisms can affect the development, growth and survival of cloned animals [23]. Despite these potential constraints, we obtained three piglets (one additional piglet died before birth). These piglets did not display abnormal phenotypes in newborn bodyweight and auxodrome before weaning.

In this short report, the transgenes were characterized by PCR, integration site analysis, Southern blotting and Western blotting. To determine the antiviral capacity of MxA against CSFV, some basic in vitro experiments were performed. We used adherent cells from tails, kidneys and umbilical cords to evaluate the antiviral activities of the MxA transgene in the pigs. The results showed that CSFV replicated poorly in the cells of all three transgenic pigs, suggesting that MxA expression markedly inhibited viral growth, but at later time points post-infection (120hpi), the level of inhibition decreased.

Fig. 2 a-b: PCR data for the neomycin resistance gene (Neo), and RT-PCR data for MxA were performed to confirm the presence of the transgene; the No. 34 clone cells were chosen for the positive control, and normal porcine tails DNA and blood RNA were chosen as negative control. 

C. Southern blotting was performed to detect the vector fragment in the genome; pGKngeoAloxp2MxA was chosen as the positive control.

D. Western blotting showed the expression of MxA in all three piglets; a cell strain stably expressing MxA was chosen for the positive control, and tissue from non-transgenic piglets was chosen as a negative control.

Fig. 3 a-c: IFA demonstrated that the proliferation of CSFV was obviously inhibited in piglet cells, including tail, kidney and umbilical cord cells. Compared with the counterparts from non-transgenic piglet, only a few MxA clone pigs’ cells displayed green fluorescence. Moreover, we found that the kidney cells were more sensitive to CSFV than the other two cell types. Our results indicate that the transgenic piglets were effectively protected by MxA.
Conclusions
A conclusion from our study is that high levels of ectopic MxA expression in transgenic swine cells do inhibit CSFV in vitro at early time points post-infection, the inhibitory effects lasted for 96 h post the infection. We expect that this new transgenic model could facilitate animal production for antiviral research targeting RNA viruses.

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Availability of data and materials
Not applicable.

Authors’ contributions
Y.C.Z. participated in conceiving the study, participated its design and coordination, helped perform the experiments, helped interpret the results. L.Y. and B.L. helped interpret the results. H.S.O.Y. and C.B.T. participated its design and coordination. Y.C.Z. participated in conceiving the study, participated its design and coordination, helped perform the experiments, helped interpret the results. T.D.W. participated its design and coordination, helped perform the experiments, helped interpret the results. H.S.O.Y. and C.B.T. participated its design and coordination. Y.C.Z. and T.D.W wrote the main manuscript, all authors read and approved the final manuscript.

Competing interests
None of the authors have any competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All animal experiments were conducted according to the guidelines for animal care and use established by the Northeast Forestry University Animal Care and Use Committee.

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