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A possible strategy to produce pigs resistant to porcine reproductive and respiratory syndrome virus

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ABSTRACT

The purpose of this study was to enhance the production of transgenic cloned embryos with porcine reproductive and respiratory syndrome virus (PRRSV) shRNA expression cassettes. To construct transgenic vector with expression targeting against PRRSV, PRRSV shRNA expression cassettes were inserted into pEGFP-N1 and the ability of resulting recombinant plasmid pEGFP-G1 inhibiting virus replication was examined in Marc-145 cells. Results showed that PRRSV replication could be significantly inhibited by pEGFP-G1 in Marc-145 cells compared with the control. The pEGFP-G1 plasmid was used to deliver a transgene expressing EGFP and the PRRSV shRNA into porcine fetal fibroblasts (PFF). Fluorescent-positive cells were used as nuclear donors for somatic cell nuclear transfer (SCNT) to produce shRNA-EGFP transgenic cloned embryos. The effects of trichostatin A (TSA) on production of transgenic cloned embryos were investigated. Reconstructed embryos were designed into 4 groups: Donor cells of Group A were treated with 50 nM TSA for 24 h before SCNT. Reconstructed embryos of Group B were treated with 50 nM TSA for 24 h after activation. Both donor cells and reconstructed embryos in Group C were treated with TSA and Group D were the control without TSA treatment. The results showed no difference ($p > 0.05$) in cleavage rates among the 4 groups; however, blastocyst developmental rates of Group B and C (30.9% and 42.0%, respectively) were higher than for Group A and D (21.2% and 22.1%, respectively) with Group C highest among groups ($p < 0.05$). Interestingly, EGFP expression intensity of transgenic cloned blastocysts of Group A was the highest. Our results provide promising evidence toward a new approach for production of transgenic cloned pigs with resistance to PRRSV and possibly a wide variety of other porcine diseases.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an infectious disease characterized by abortion in pregnant sows, respiratory distress in piglets and growing pigs, and high mortality in piglets. Since it was first identified in the USA in 1987 and subsequently in Europe (Ait-Ali et al., 2007), this disease has resulted in significant economic losses to the swine industry in many pig-producing countries, such as China, Laos, Thailand and USA (Li et al., 2007a,b, 2012; Thanawongnuwech et al., 2004; Neumann et al., 2005). Current vaccine strategies to control the disease are still a challenge (Meng, 2000; Mengeling et al., 2003; Opriessnig et al., 2002). To date, no efficient method is available to prevent and control the disease, and thus exploration of new antiviral strategies is important.

RNAi is a conserved gene silencing mechanism to double-stranded RNA, which mediates the sequence-specific degradation of homologous mRNA in both endogenous parasitic and exogenous pathogenic nucleic acids (Golding et al., 2006). Recently, RNAi technology has been used to induce gene silencing in a number of viruses, including porcine circovirus type 2 (PCV-2) (Feng et al., 2008), porcine encephalomyocarditis virus (Jia et al., 2008), foot-and-mouth disease virus (Chen et al., 2004), Marek's disease virus (Chen et al., 2008), hepatitis C virus (HCV) (Kapadia et al., 2003), prototypic arenavirus lymphocytic choriomeningitis virus (Sanchez et al., 2005), influenza virus (Thomas et al., 2005; Lyall et al., 2011), avian reovirus (Ji et al., 2008) and classical swine fever virus (Porntrakulpipat et al., 2010) in vivo or in vitro.

RNAi can be combined with transgenic technology to produce transgenic animals with the resistance against some diseases. Several examples of the production of transgenic animal via combination of these technologies have been reported; these results demonstrated that gene constructs expressing shRNA can be stably incorporated into chromosomes of the nuclear donor cell and used to create transgenic animals in which target genes are inhibited. Goats and cattle expressing prion protein (PrP) shRNA (Golding et al., 2006) and pigs expressing porcine endogenous retrovirus (PERV) shRNA (Dieckhoff et al., 2008; Ramsoondar et al., 2009) are among most compelling examples.

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Recently specific shRNA directed against ORF5 and ORF7 regions of PRRSV were shown to inhibit PRRSV replication in Marc-145 cells (Huang et al., 2006; Li et al., 2007a). The purposes of this study were first to construct a pEGFP-G1 transgenic vector that expresses shRNA targeting ORF5 of PRRSV and is suitable for SCNT and selection and ultimately to assess the ability to generate transgenic cloned blastocysts.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich™ unless otherwise stated.

2.1. Construction of vector expressing PRRSV shRNA

The pSUPER–shRNA plasmids (pSUPER-G1 and pSUPER-mG1; Fig. 1) were kindly provided by Dr. Ping Jiang (Huang et al., 2006). Plasmid pSUPER-mG1 harbored 3 different nucleotides compared with pSUPER-G1 and was used as the negative control. pSUPER–shRNA plasmids were used as a template to produce shRNA-expression cassettes by PCR and to generate EcoO109I restriction enzyme sites (AGGCCCT). shRNA-expression cassettes were inserted into the EcoO109I site of pEGFP-N1 (Fig. 1). The recombinant plasmid constructs were identified with EcoO109I restriction enzymes (TaKaRa) digestion, confirmed by sequencing (Invitrogen) and designated as pEGFP–shRNA (pEGFP-G1, pEGFP-mG1, respectively; Fig. 1). pEGFP–shRNA used for transfection was purified with the EndoFree Maxi Plasmid Kit (TianGen Biotech Co., Ltd., Beijing, China).

2.2. Cell transfection and viral challenge assays

Marc-145 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL) supplemented with 10% (v/v) heat-inactivated newborn calf serum (NCS, Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were used to assess the expression of shRNA and challenge with PRRSV. Marc-145 cells were seeded and incubated overnight in 24-well plates at 37 °C with 5% CO_2 before transfection. When cells reached 60–70% confluency, they were transfected with plasmid (pEGFP-G1, pEGFP-mG1) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Twenty-four hours after transfection, the transfection efficiency was detected by monitoring the numbers of EGFP fluorescent-positive cells under an inverted fluorescent microscope. Subsequently, transfected cells were infected with Chinese PRRSV S1 strain (Huang et al., 2006) at multiplicity of infection (moi) of 10 and viral infections were allowed to proceed for indicated time.

2.3. Preparation of nuclear donor cells

Porcine fetal fibroblasts (PFF) were isolated from porcine fetuses on Day 35 of gestation. Fetuses were rinsed 3 times with Dulbecco's phosphate-buffered saline (PBS). After removal of limbs, head, heart, liver, and intestine, the remnants were finely minced into small pieces (1 mm^3) using scissors in PBS. Minced pieces were dissociated with collagenase (200 IU /ml) dissolved in DMEM supplemented with 15% (v/v) fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin for 24 h at 38.5 °C and 5% CO_2 in air. After centrifugation at 800×*g* for 5 min, the cell pellet was resuspended in cell culture medium with the density of 10^4/ml and seeded into culture flask. When reaching 80% confluence, subculture was conducted by trypsinization for 5 min using 0.25%Trypsin–EDTA solution. After 2 passages, the cells were resuspended and diluted to10^7/ml in freezing medium consisting of 70% (v/v) DMEM, 10% (v/v) dimethyl sulfoxide (DMSO) and 20% (v/v) FBS and transferred to a freezing container at -80 °C overnight. Cells were transferred into liquid nitrogen next morning for long-term storage (Lai and Prather, 2003).

Before transfection, frozen PFF were thawed and seeded in a 12-well plate to reach 70%-80% confluency on the day of transfection. Cells in each well were transfected with 1 μg pEGFP-G1 and 10 μl FuGENE® HD transfection reagent (Roche) following the manufacturer's protocol. Twenty-four hours after transfection, cells expressing EGFP were observed under a fluorescence microscope. Transfected cells were trypsinized and seeded in a culture dish. After 24 h of culture, cells were selected with 600 μg/ml G418 (Invitrogen). After culture for 10 d, G418-resistant colonies expressing EGFP (Fig. 2) were isolated separately and seeded in a 48-well plate after trypsinization to expand the transgenic cell population. PRRSV shRNA were tested by PCR. Only recombinant colonies carrying shRNA–EGFP were frozen and conserved in liquid nitrogen for further use in SCNT.

2.4. Preparation of recipient oocytes

Collection of oocytes and in vitro maturation (IVM) was as previously described (Wu et al., 2006). Porcine ovaries were collected at a local slaughterhouse and within 2 h transported to the laboratory in physiological saline at 37 °C. Follicular fluid and cumulus-oocyte complexes (COC) from follicles between 2 and 5 mm in diameter were aspirated with a 12-gauge needle attached to a 10 ml disposable sterile syringe. The resulting COC with 3 or more layers of compact cumulus cells were chosen for the experiment and washed 3 times with PBS supplemented with 0.01% polyvinyl-alcohol (PVA). The IVM medium for oocytes was TCM 199 (Gibco) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.1% PVA (w/v), 0.1 mg/ml cysteine, 10 IU/ ml hCG (Ningbo Hormonal Reagents Co., Ltd., Zhejiang, China), 10 IU/ml PMSG (Tianjin Huafu High & New Biotech Co., Tianjin, China), 10 ng/ml epidermal growth factor (EGF), 75 mg/ml penicillin, 50 mg/ml

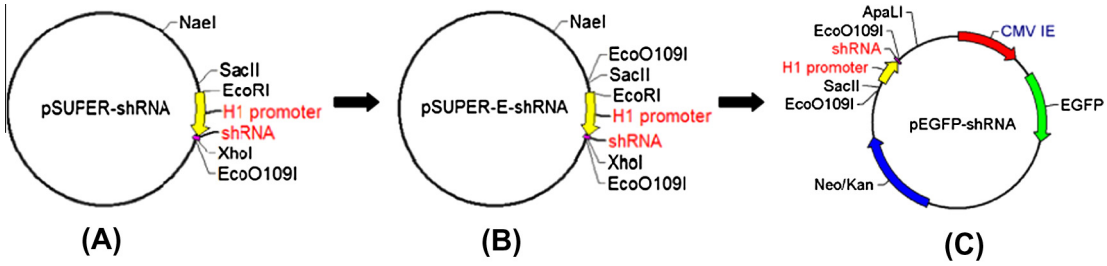

Fig. 1. Construction of vector expressing shRNA-EGFP. (A) Structure of pSUPER–shRNA. (B) Create EcoO109I restriction sites at 5′ terminal of the shRNA-expression cassettes on pSUPER–shRNA. (C) Structure of recombinant plasmid pEGFP–shRNA.

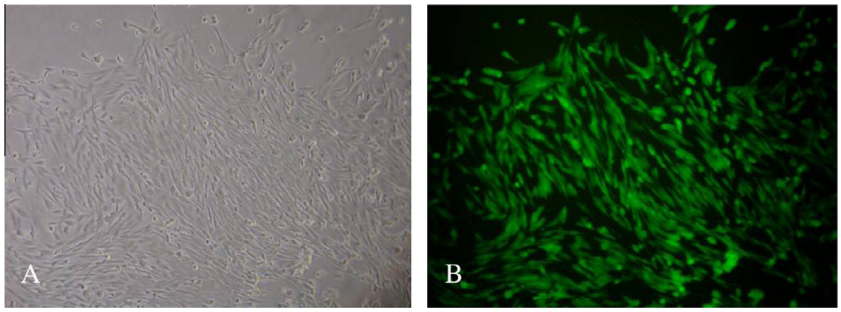

Fig. 2. Stable transgenic colonies expressing EGFP (100×). (A) Bright-field images. (B) Fluorescent images under UV light.

streptomycin and 10% (v/v) porcine follicular fluid (pFF, self-made). Each group of 80–100 COC was placed into one well of a 4-well multidish (Nunclon, Roskilde, Denmark) containing 500 μl of IVM medium pre-equilibrated for at least 4 h, and cultured at 38.5 °C and 5% CO_2 in air (100% humidity) for 22 h. Then the COC were transferred into PMSG-/hCG-free IVM medium and cultured for another 20 h. Mature COC were treated with 1 mg/ml hyaluronidase for 5 min to remove cumulus cells by repeated pipetting. Only oocytes with uniform cytoplasm intact cell membrane and with first polar body (pbI) extruded into the perivitelline space were used for SCNT operations.

2.5. Nuclear transfer

The transgenic nuclear donor cells were thawed and cultured routinely and then trypsinized to harvest individual cells. SCNT procedures were conducted as previously described (Ju et al., 2010). Briefly, oocytes and nuclear donor cells were prepared in microdrops of manipulation medium (Hepes-buffered TCM199 with 3 mg/ml BSA) supplemented with 7.5 μg/ml cytochalasin B. After incubation for 15 min in the incubator, pbI together with 10–20% of adjacent cytoplasm of the oocytes were removed by aspiration with a glass pipette needle. A single intact fluorescent cell was injected into the perivitelline space through the same slot and placed adjacent to the recipient cytoplasm (Fig. 3). After being washed 3× in fresh warm porcine zygote medium-3 (PZM-3) with 3 mg/ml BSA (Lai and Prather, 2003), the reconstructed oocytes were placed into pre-equilibrated PZM-3 for 1.5 h until fusion and activation. The fusion and activation were accomplished simultaneously with a single DC pulse of 1.5 kV/cm for 40 μs provided by an electro-cell manipulator (CRY-3, Ningbo Xinzhi Co., Ltd, Ningbo, China) in fusion medium containing 0.5 mM Hepes, 0.3 M mannitol, 1.0 mM $CaCl_2$ and 0.1 mM $MgCl_2$. Subsequently, the reconstructed oocytes were washed 3× in fresh warm PZM-3 incubated in PZM-3 for 30 min, and evaluated for fusion under an inverted fluorescence microscope.

2.6. Embryo culture and blastocyst quality evaluation

Only fused embryos expressing EGFP were cultured (Fig. 3). Each group of 30–50 embryos was placed into one well of a 4-well cell culture plate containing 500 μl PZM-3 covered with dimethylpolysiloxane (DMPS), which was cultured at 38.5 °C and in 5% CO_2 and 100% humidity for 7 d after activation. Activation was conducted on Day 0; cleavage and blastocyst formation were counted at Day 2 and Day 7, respectively. Cleavage rates, blastocyst rates and number of nuclei were used to evaluate in vitro development. The expression of the reporter gene EGFP in transgenic cloned blastocysts was observed under UV light and used as an evaluation of embryos carrying PRRSV shRNA.

Number of nuclei was counted in expanded blastocysts that approached hatching. Following a previous report (Zhang et al., 2007), blastocysts selected for cell number count were fixed for 10 min in PBS containing 4% paraformaldehyde. After 3 washes in PBS, fixed blastocysts were transferred into PBS containing 10 μg/ml Hoechst 33342, incubated in darkness for 15 min at room temperature. After 10 washes in PBS, blastocysts were placed on a glass slide, excessive liquid was removed, and blastocysts were mounted with a drop of antifade polyvinylpyrrolidone mounting medium (Beyotime Institute of Biotechnology, Jiangsu, China). Subsequently, a coverslip was gently pressed to stretch cells, which were observed and photographed under an inverted fluorescence microscope.

2.7. TSA treatment

Transgenic donor cells before nuclear transfer and cloned embryos after activation were treated with or without 50 nM TSA for 24 h (Zhang et al., 2007; Bo et al., 2011). Group A was only donor cells treated with TSA, Group B only cloned embryos treated with TSA, Group C was both donor cells and cloned embryos treated with TSA, and Group D was neither donor cells nor cloned embryos were treated with TSA. Group D was designated the control.

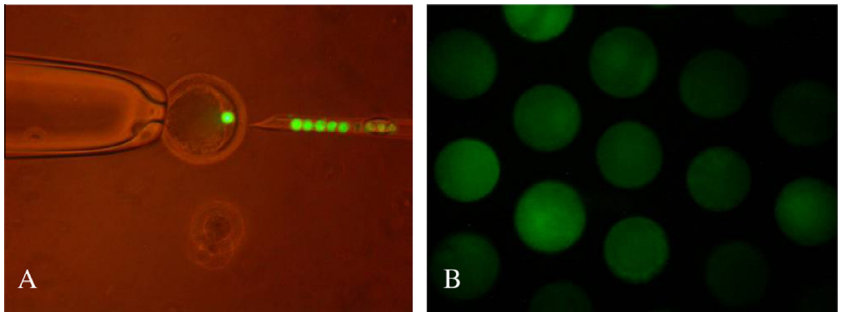

Fig. 3. Production of reconstructed embryos (200×). (A) Injection of transgenic cells into enucleated oocytes. (B) Reconstructed embryos after fusion for 30 min.

2.8. Real-time RT-PCR

To compare EGFP expression intensity in blastocysts with different TSA treatments, real-time RT-PCR was performed. Total RNA was extracted from 30 blastocysts from each group using TRIzol reagent (Invitrogen), according to the manufacturer's instruction. RNA from each sample was converted to cDNA by using PrimeScript™ RT Master Mix (TaKaRa) according to manufacturer's instructions. Real-time RT-PCR was performed using the SYBR Premix Ex Taq™ (TaKaRa) and the 7300 Real-Time PCR System (Applied Biosystems) as previously described (Ju et al., 2010) with the following parameters: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 31 s. For each cDNA sample, both target and reference genes were amplified independently on the same plate and in the same experimental run in triplicate. Results of real-time PCR were analyzed using $2^{-\Delta\Delta CT}$ method to compare the relative transcriptional levels of the target genes in each sample (Livak and Schmittgen, 2001).

2.9. Statistical analysis

All experiments were repeated at least 3×. Data of cleavage rate, blastocyst rate expressed as proportions (percentages), and cell number per blastocyst were presented as mean ± SEM values. The percentages were subjected to an arc-sine transformation. All datas were analyzed by one-way ANOVA with SPSS software version 19.0. Comparisons of mean values among treatments were performed using multiple comparison test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Inhibition of PRRSV replication in MARC-145 cells

To assess the capacity of recombinant plasmid pEGFP-G1 to inhibit PRRSV replication in vitro, pEGFP-G1 and pEGFP-mG1 were transfected into Marc-145 cells (Fig. 4). At 60 h post-infection, only PRRSV-infected harbored extreme CPE effect and cells detached significantly from the monolayer (Fig. 4C). Only cells transfected with pEGFP-G1 (Fig. 4 A) were visibly more resistant to PRRSV infection than with pEGFP-mG1 (Fig. 4B). The CPE effect of cells transfected with pEGFP-mG1 was also similar to that only PRRSV-infected. Taken together these data suggest that pEGFP-G1 is effective in hampering PRRSV infection in vitro.

3.2. Effect of trichostatin A on the developmental competence of the transgenic cloned embryos in vitro

To enhance the production of transgenic cloned embryos, the effect of a histone deacetylation inhibitor TSA was assessed after SCNT. As shown in Table 1, was no significant difference among the cleavage rates of transgenic cloned embryos from 4 treatments with TSA ($p > 0.05$); the transgenic cloned blastocyst rate of Group C was significantly higher than that of Group A, B and the control (42.0% versus 21.2%, 30.9% and 22.1%, respectively; $p < 0.05$). In addition, blastocyst rate of Group B was also notably higher than that of Group A ($p < 0.05$), but no difference was seen between Group A and the control ($p > 0.05$). No effect of TSA treatment was observed for average cell numbers per blastocyst ($p > 0.05$; Table 1, Fig. 5).

3.3. Effect of trichostatin A on the EGFP expression intensity in transgenic cloned blastocysts

Observed under UV light, the expression intensities of EGFP in transgenic cloned blastocysts of Groups A and C (Fig. 6 A, C) was higher than those from Group B and D (Fig. 6 B, D). EGFP mRNA expression in blastocysts with TSA treatment was significantly higher than that without TSA treatment ($p < 0.05$; Fig. 7). Interestingly, EGFP mRNA expression analysis showed that TSA treatment of donor cells and/or embryos could significantly increase EGFP expression intensity, and also the donor cells or both donors and embryos treated with TSA (Groups A and C) provided greater expression intensities than only embryos treated with TSA (Group B, $p < 0.05$; Fig. 7).

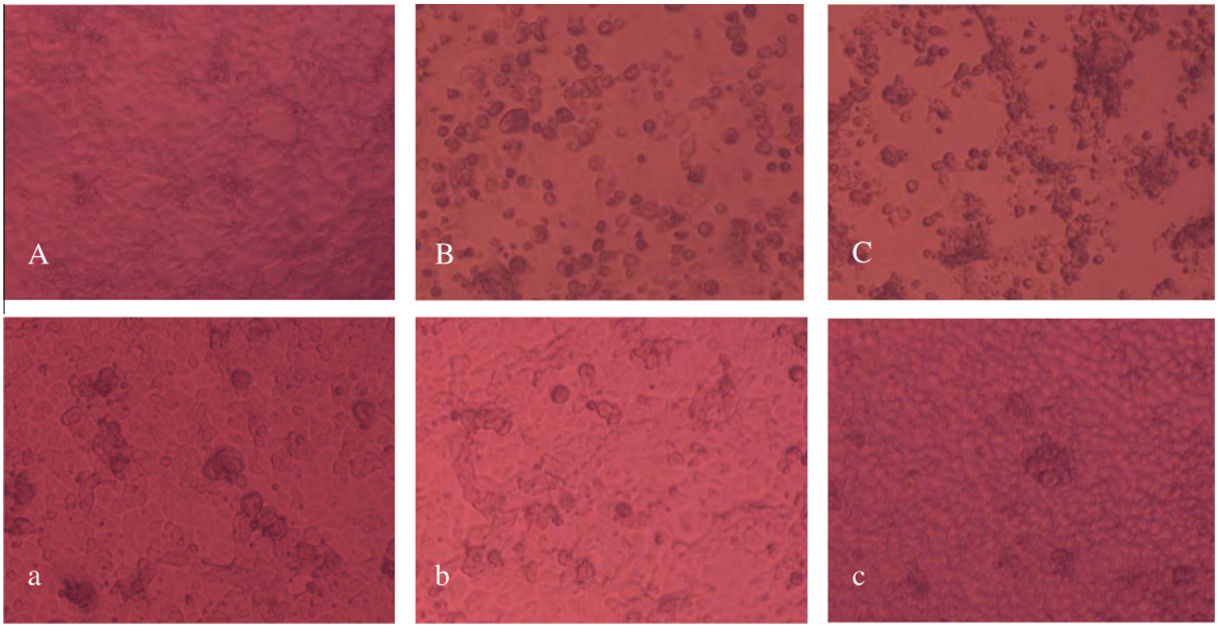

Fig. 4. Cytopathic effect analysis of PRRSV infection on Marc-145 cells at 60 h post-infection (100×). Cells were seeded in 24-well plates in triplicate. At 24 h post-transfection, cells were infected with PRRSV and cytopathic effects (CPE) were monitored. Accordingly, cells only transfected with plasmid were used as plasmid controls. Cells were infected with PRRSV from Group A to C, and non-infected cells from Groups a–c. (A, a) pEGFP-G1. (B, b) pEGFP-mG1. (C, c) No plasmids.

Table 1
Effect of trichostatin A on the development in vitro of porcine transgenic cloned embryos.

Treatment group	Donor cells with TSA (nM)	Embryos with TSA (nM)	No. of embryos cultured	No. of embryos cleaved (%)	No. of blastocysts (%)
A	50	0	99	74 (74.7)	21 (21.2)[a]
B	0	50	81	68 (84.0)	25 (30.9)[b]
C	50	50	88	78 (88.6)	37 (42.0[c]
Control	0	0	95	73 (76.8)	21 (22.1)[a]

Different superscripts in the same column indicate significant differences ($p < 0.05$).

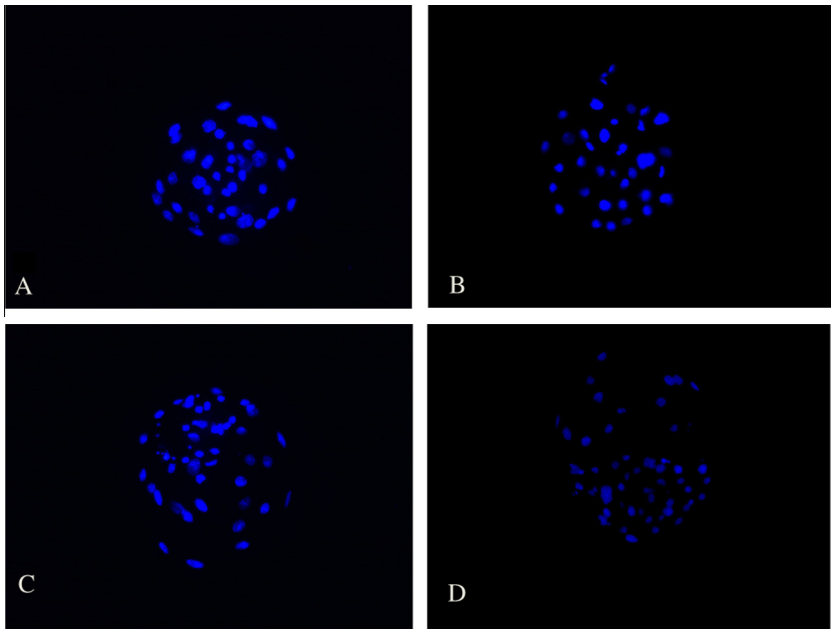

Fig. 5. Number of nuclei in transgenic cloned blastocysts using different TSA treatment was determined after staining with Hoechst 33342 (200×). A, B, C and D represent the corresponding group.

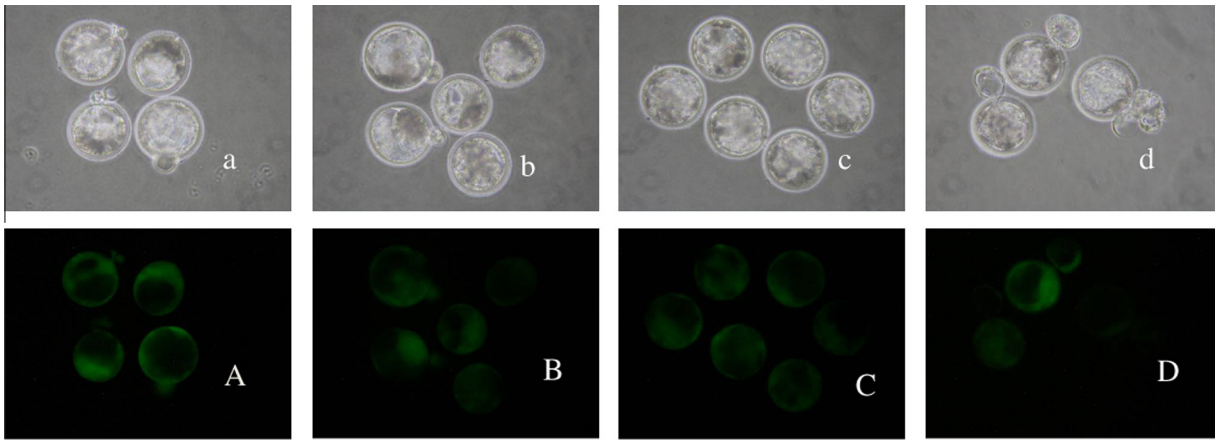

Fig. 6. Expression of fluorescent EGFP in transgenic cloned blastocysts with different TSA treatment (200×). The embryos in micrographs under UV light (A–D); the same embryos in micrographs under bright light (a–d).

4. Discussion

Genetic engineering of animals has played a pivotal role in research for agriculture, biomedicine, physiology and genetics. To date, genetically modified animals have been produced in several mammals. RNAi technology has been developed as a powerful tool to knockdown the expression of host genes or exogenous sequences of nucleic acids (Morris et al., 2004). Recently, RNAi has been recognized as a versatile therapeutic or prophylactic methodology to combat viruses (Haasnoot et al., 2003). In the present experiments, we produced porcine transgenic cloned embryos with potential expression of shRNA targeting against PRRSV, an important first step toward efficient production of transgenic pigs resistant to PRRSV infection. Furthermore, it is tempting to suggest that this approach may be suitable for creation of transgenic pigs resistant to a wide range of infectious diseases triggered by pathogens.pEGFP-N1 plasmid has been widely used as transgenic vector that can be stably incorporated into the genome of mammalian

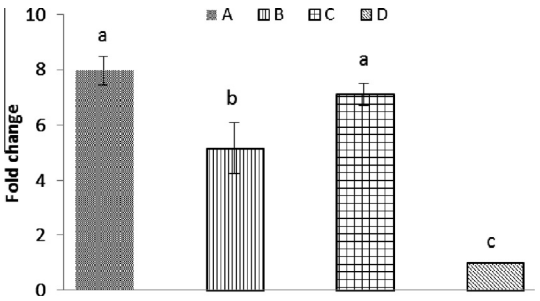

Fig. 7. Relative EGFP mRNA expression patterns in blastocysts from four treatments with TSA. Different superscripts indicate significant differences ($p < 0.05$).

cells (Hyun et al., 2003). Indeed, the fluorescent EGFP reporter gene driven by cytomegalovirus (CMV) promoter has often been used as a versatile selection marker to create transgenic donor cells for SCNT as well as to evaluate embryos and animals that carry a transgene. We have designed and constructed pEGFP-G1 plasmid that expresses shRNA according to the construct pSUPER-G1 as previously reported (Huang et al., 2006). We selected pSUPER-G1 because of the expression of a shRNA (He et al., 2007), which facilitated selection of stable integrations in transgenic PFF to be used as nuclear donors for SCNT and allow shRNA expression to be monitored under an inverted fluorescence microscope. First, we constructed transgenic plasmids pEGFP-G1 expressing shRNA targeting against the GP5 protein gene of PRRSV, and examined the feasibility of inhibition of the PRRSV replication in Marc-145 cells. Previous results demonstrated that the shRNA-expression cassettes targeting against PRRSV could inhibit virus replication by detection of CPE, $TCID_{50}$, IFA, RT-PCR (Huang et al., 2006). Therefore, CPE was a direct method that demonstrated whether pEGFP-G1 could inhibit PRRSV replication. In this study, pEGFP-G1 resulted in a significant reduction in viral replication by CPE observed; moreover, the cells transfected only with plasmid pEGFP-G1 proliferated normally. Therefore, pEGFP-G1 could be used to deliver a safely transgene and significantly inhibit PRRSV replication in cells.

The eventual aim of this study is to generate porcine transgenic cloned pigs resistant to PRRSV via RNAi-based and SCNT methodologies. To improve the likeliness of generating EGFP-expressing blastocysts, TSA was employed. Recent reports have shown that TSA can result in higher preimplantation embryonic development of SCNT embryos in pigs (Zhang et al., 2007; Li et al., 2008), cattle (Ding et al., 2008; Iager et al., 2008), and rabbits (Shi et al., 2008). In addition, it was also confirmed that 8 CpG sites of the CMV promoter are regulated by methylation (Krishnan et al., 2006), and CMV promoter-controlled transgenes could be activated with TSA (Choi et al., 2005). Finally, TSA was shown to increase EGFP expression from donor cells to morula stage (Bo et al., 2011).

The effect of TSA on in vitro developmental competence of the transgenic SCNT embryos, especially EGFP expression in the blastocyst stage, was investigated. Our results showed that transgenic SCNT embryos after activation with TSA (Table 1, Groups B and C) improved cloning efficiency compared with mock controls (Table 1, Groups A and D). It is thought that TSA can induce hyperacetylation of the core histones after nuclear transfer, as well as enhance DNA demethylation of the genome, which is a necessary part of genetic reprogramming (Zhao et al., 2009). In addition, TSA treatment of transgenic donor cells before SCNT (Groups A and C) enhanced EGFP expression in the blastocyst stage compared with the controls without TSA treatment (Group B and the control). It is probable that TSA, as a histone deacetylase inhibitor, can prevent DNA methylation, increase histone acetylation, reactivate and maintain EGFP expression in the promoter region from these transgenic donor cells to blastocyst stage. However, it was demonstrated that TSA treatment of donors or both donors and embryos showed higher EGFP mRNA expression than TSA treatment to only embryos. This observation suggests that exogenous transgene expression may be derived from TSA treatment to somatic cells more effectively than with TSA treatment to embryos. Our results suggest that TSA treatment can improve cloning efficiency and enhance EGFP expression.

With the manipulation procedures and culture systems described here, all embryos expressing high EGFP exhibited uniform fluorescence throughout the inner cell mass and trophectoderm. Moreover, the in vitro developmental competence of the shRNA–EGFP transgenic cloned embryos, cleavage rates, and mean cell numbers per blastocyst were similar to values reported previously (Zhao et al., 2009; Cao et al., 2012).These observations indicate that shRNA–EGFP transgene was incorporated into the zygotic genome via SCNT. Given the nature of our gene construct, expression of EGFP is indicative that shRNA targeting PRRSV was also expressed. One of our goals was to determine the developmental competence of porcine transgenic cloned embryos. These results suggest that the methods used here are effective in the production of porcine transgenic cloned embryos containing an expression cassette eliciting RNAi-based silencing. Some groups have reported that TSA treatment has various detrimental effects on in vitro and in vivo development of SCNT embryos (Meng et al., 2009; Wu et al., 2008; Svensson et al., 1998; Tsuji et al., 2009), which provides a useful experimental platform to produce transgenic animals efficiently. We have also confirmed novel histone deacetylation inhibitor with hypotoxicity.

In summary, for the first time we produced porcine transgenic cloned embryos potentially resistant to PRRSV using vector-based shRNA methodology. Further study is needed to investigate whether such a strategy could protect against PRRSV infection in porcine transgenic cloned embryos or offsprings.

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