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Production of germline transgenic pigs co-expressing double fluorescent proteins by lentiviral vector

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ABSTRACT

Genomic integration of transgene by lentiviral vector has been proved an efficient method to produce single-transgenic animals. But it failed to create multi-gene transgenic offspring. Here, we have exploited lentivirus to generate the double-transgenic piglets through the female germline. The recombinant lentivirus containing fluorescent proteins genes (*DsRed1* and *Venus)* were injected into the perivitelline space of 2-cell stage *in vitro* porcine embryos.

Compared to control group, there was no significantly decreased in the proportion of blastocysts, and the two fluorescent protein genes were co-expressed in almost all the injected embryos. Total of 32 injected *in vitro* embryos were transferred to 2 recipients. One recipient gave birth of three live offspring, and one female piglet was identified as genomic transgene integration by PCR analysis. Subsequently, the female transgenic founder was mated naturally with a wild-type boar and gave birth of two litters of total 23 F(1) generation piglets, among which *Venus* and *DsRed1* genes were detected in 11 piglets and 10 kinds of organs by PCR and RT-PCR respectively. The co-expression of two fluorescent proteins was visible in four different frozen tissue sections from the RT-PCR positive piglets, and 3 to 5 copies of the transgenes were detected to be integrated into the second generation genome by southern blotting analysis. The transgenes were heritable and stably integrated in the F(1) generation. The results indicated for the first time that lentiviral vector combined with natural mating has the potential to become a simple and practical technology to create germline double-transgenic livestock or biomedical animals.

1. Introduction

Genetically modified livestock have important implications for agricultural or biomedical research. It has special significance for the transgenic pig research, which is expected to improve animal production traits, build genetic disease models, screen vaccines, or produce xenogeneic organs (Clark and Whitelaw 2003; Saeki et al., 2004). The first transgenic livestock were produced by pronuclear microinjection of DNA into zygotes in sheep and pig (Hammer et al., 1985). Subsequently, transgenic sheep and cattle were born by somatic-cell nuclear transfer (SCNT) techniques (Schnieke et al., 1997; Cibelli et al., 1998). In order to overcome the disadvantages of microinjection and SCNT, including complicated operation, high cost, inefficiency, *etc*., lentiviral vector has been used to deliver the exogenous genes to livestock (Hofmann et al., 2003). The first lentiviral transgenic pig was produced by injecting lentivirus into zygotes, and had generation rates of between 19 and 33% (Hofmann et al., 2003), signifi-

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cantly higher than the 1% obtained by pronuclear DNA microinjection (Hammer et al., 1985). Lentiviral vectors could efficiently deliver transgenes into a host cell genome, but almost all of the lentiviral transgenesis studies were confused on the transfer of a single exogenous gene (Lois et al., 2002; Hofmann et al., 2003; Reichenbach et al., 2010; Liu et al., 2013). It is difficult to generate multi-transgenic animals using lentiviral technologies. However, strategies for expressing of multiple foreign genes were often needed in establishing animal disease models, gene therapy protocols, xenotransplantation, transgenic livestock, *etc*. It was not clear whether the lentiviral vector could efficiently deliver multiple exogenous genes to recipient genomes simultaneously. At the same time, we also wished to assess whether the multiple transgenes could be passed on to the second generation offspring. Considering the efficiency in exogenous genes transferring into the host genomes, this study aimed to produce double-transgenic pigs by perivitelline injection of the multigenes lentivirus, and to explore whether the multiple transgenes could be transferred to the next generation offspring by germ cells.

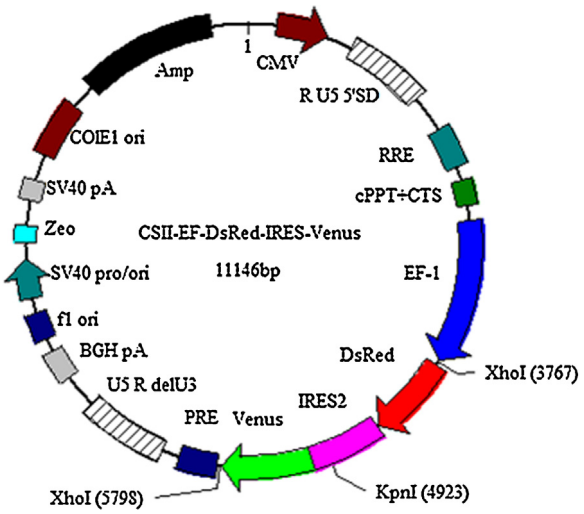

Fig. 1. Map of lentiviral vector.

2. Materials and methods

2.1. Animals

All animals in this study were DLY tri-specific hybrid pigs from Haining pig farm of the Zhejiang Academy of Agricultural Sciences (Hangzhou, PR of China). All studies performed in pigs were approved by the Committee of Animal Research and Use, and all pigs were treated humanely.

2.2. Production of lentiviral virus

The *DsRed1* gene was amplified from a pDsRed1-N1 plasmid (Clontech, Palo Alto, CA) using Pfu DNA Polymerase (Promega, Southampton, UK), with the special primers containing *Not*I and *Bam*HI restriction enzyme sites. The amplified *DsRed1* gene was inserted into the lentiviral plasmid pCSII-EF-MCS-IRES2-Venus (RIKEN BRC, Japan), and renamed pCSII-EF-DsRed1-IRES2-Venus (Fig. 1). Recombinant lentivirus was produced as previously described with slight modification (Lee et al., 2009). Briefly, the transfer plasmid (pCSII-EF-DsRed1-IRES2-Venus), the packaging plasmid (pCAG-HIVgp) and the envelope plasmid (pCMV-VSV-G-RSV-Rev) were co-transfected into 293 T cells using calcium phosphate transfection buffer. The virus-containing media were collected 3 to 4 days after co-transfection, and filtered through a 0.45 μm filter, followed by centrifugation at 50,000*g* for 2 h. The virus particle pellet was resuspended by pipetting in an appropriate volume of Hanks' Balanced Salt Solution (HBSS, GIBCO).

2.3. Porcine embryo collection and virus injection

Porcine embryos were obtained by *in vitro* matured/fertilized oocytes and *in vivo* fertilized embryos. The *in vitro* maturation/*in vitro* fertilization (IVM/IVF) was performed according to previously established methods (Iwamoto et al., 2005; Yoshioka et al., 2008). In brief, porcine oocytes fertilized *in vitro* were cultured in PZM-5 medium (IFP, Japan) for 24 h at 39 °C in 5% O_2 and 5% CO_2 in humidified compressed air to acquire two-cell stage embryos. In order to obtain *in vivo* embryos, the donors were superovulated with 1500 IU pregnant mare serum gonadotropin (eCG, Ningbo Sangsheng, China) from day 13–15 during the estrous cycle, and ovulation was stimulated with 600 IU human chorionic gonadotropin (hCG, Ningbo Sangsheng, China) 3 days later. During the following 24–36 h, the donor animals were mated naturally twice and the 2-cell embryos were recovered at 33 h after the first mating by flushing the oviducts with PBS(−) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), and 50 mg/ml gentamicin sulfate (Sigma, St. Louis, MO, USA) in 38 °C. *In vitro/in vivo* porcine embryos were cultured in PZM-5 and approximately 50 pl of lentivirus was injected into the perivitelline space of 2-cell embryos using glass capillaries connected to a micromanipulator (TE2000-U, Nikon, Japan).

2.4. Porcine embryo transfer

Six-month-old prepubertal gilts were used as recipients and previously synchronized by injection of 30 mg altrenogest per recipient (Prostaglandin, Ningbo Sangsheng, China) on the twelfth day after the last estrus, followed by an injection of 1000 IU eCG on the day after the last injection of altrenogest. Ovulation was induced 3 days later with 600 IU hCG. Embryo transfer was performed surgically under general anaesthesia that was induced with injection of ketamine hydrochloride (5 mg/kg BW IM, Shenyang Veterinary Drugs, China) and maintained with isoflurane (Heibei, Jiupai, China) in oxygen using a rebreathing anaesthetic machine. Two-cell porcine embryos were transferred to recipients within 1 h after lentivirus injection. For each recipient, injected embryos were transferred bilaterally into the uterine horns.