Open Access
Asian Australas. J. Anim. Sci.
Vol. 29, No. 7 : 925-937 July 2016
http://dx.doi.org/10.5713/ajas.15.0505
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

Production of Transgenic Pigs with an Introduced Missense Mutation of the Bone Morphogenetic Protein Receptor Type IB Gene Related to Prolificacy

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ABSTRACT: In the last few decades, transgenic animal technology has witnessed an increasingly wide application in animal breeding. Reproductive traits are economically important to the pig industry. It has been shown that the bone morphogenetic protein receptor type IB (BMPR1B) A746G polymorphism is responsible for the fertility in sheep. However, this causal mutation exits exclusively in sheep and goat. In this study, we attempted to create transgenic pigs by introducing this mutation with the aim to improve reproductive traits in pigs. We successfully constructed a vector containing porcine BMPR1B coding sequence (CDS) with the mutant G allele of A746G mutation. In total, we obtained 24 cloned male piglets using handmade cloning (HMC) technique, and 12 individuals survived till maturation. A set of polymerase chain reactions indicated that 11 of 12 matured boars were transgene-positive individuals, and that the transgenic vector was most likely disrupted during cloning. Of 11 positive pigs, one (No. 11) lost a part of the terminator region but had the intact promoter and the CDS regions. cDNA sequencing showed that the introduced allele (746G) was expressed in multiple tissues of transgene-positive offspring of No.11. Western blot analysis revealed that BMPR1B protein expression in multiple tissues of transgene-positive F_1 piglets was 0.5 to 2-fold higher than that in the transgene-negative siblings. The No. 11 boar showed normal litter size performance as normal pigs from the same breed. Transgene-positive F_1 boars produced by No. 11 had higher semen volume, sperm concentration and total sperm per ejaculate than the negative siblings, although the differences did not reached statistical significance. Transgene-positive F_1 sows had similar litter size performance to the negative siblings, and more data are needed to adequately assess the litter size performance. In conclusion, we obtained 24 cloned transgenic pigs with the modified porcine BMPR1B CDS using HMC. cDNA sequencing and western blot indicated that the exogenous BMPR1B CDS was successfully expressed in host pigs. The transgenic pigs showed normal litter size performance. However, no significant differences in litter size were found between transgene-positive and negative sows. Our study provides new insight into producing cloned transgenic livestock related to reproductive traits. (**Key Words:** Transgenic, Handmade Cloning, Pig, *BMPR1B*, Coding Sequence, Reproductive Traits)

INTRODUCTION

Since the emergence of the world's first transgenic animals (Gordon et al., 1980), animal transgenic techniques have attracted researchers' interest for decades. A number of transgenic animal methods like pronuclear microinjection, viral-mediated DNA transfer and sperm-mediated DNA transfer have been developed, each of which has its advantages and disadvantages. Somatic cell nuclear transfer (SCNT) is one of the most extensively used techniques to obtain genetically modified domestic animals (Yang and Ross, 2012). More recently, a novel SCNT technique called handmade cloning (HMC), has been established to improve the efficiency of animal cloning (Vajta et al., 2004; Du et al., 2007; Zhang et al., 2012a). In the past 2-3 years, transcription activator-like effector nucleases and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases have become powerful tools in genome engineering (Gaj et al., 2013).

Transgenic technology has been applied in pigs (Prather

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Submitted Jun. 10, 2015; Revised Sept. 23, 2015; Accepted Oct. 25, 2015

et al., 2008) after the birth of the first transgenic livestock animal (Hammer et al., 1985). Since the pig is an excellent model for human medical and disease research, transgenic pigs have been generated to study disease related traits, such as clinical xenotransplantation (Cho et al., 2011; Yeom et al., 2012), pharmaceuticals (Watanabe et al., 2012), and models of human diseases (Renner et al., 2010; Yang et al., 2010; Sommer et al., 2011). In comparison, fewer studies focused on traits related to production (Hammer et al., 1985), meat quality (Lai et al., 2006) and low-phosphorus waste (Golovan et al., 2001).

Reproductive traits are economically important in the pig industry. However, only one transgenic study has been reported to improve pig reproductive traits (gilt follicular development). Guthrie et al. introduced human *Bcl-2* gene affecting follicular growth into the pig genome (Guthrie et al., 2005). Nevertheless, the transgenic gilts did not decrease the follicular atresia or increase ovulation rate. Bone morphogenetic protein receptor, type IB (*BMPR1B*) encodes a member of the bone morphogenetic protein receptor family of transmembrane serine/threonine kinases, which are involved in endochondral bone formation and embryogenesis. The *A746G* (*FecB*) mutation in *BMPR1B* significantly affects the ovulation rate in Booroola-Merino sheep (Mulsant et al., 2001; Souza et al., 2001). The ewes with heterozygous and homozygous *FecB* mutation produced about 1.5 and 3.0 extra ova per oestrus, resulting in approximately 1.0 and 1.5 extra lambs, respectively (Davis, 2005). However, this causal mutation has not been found in mammals other than sheep and goat (Chu et al., 2010; Chu et al., 2011).

In this study, we constructed a vector containing the porcine *BMPR1B* CDS with the mutant allele (*G*) at the *FecB* site by site-directed mutagenesis, and then introduced the vector into primary porcine fetal fibroblasts (PFF) by liposome-mediated transfection. Then we performed HMC to produce transgenic boars, with the aim to improve reproductive traits.

MATERIALS AND METHODS

Ethics statement

All procedures involving animals followed the guidelines for the care and use of experimental animals approved by the State Council of the People's Republic of China. The ethics committee of Jiangxi Agricultural University specifically approved this study.

Detection of the *BMPR1B A746G* mutation in diverse pig breeds

DNA samples of 20 pigs (10 females and 10 males) each from 3 Western breeds (Large White, Landrance, and Duroc), 11 Chinese local breeds (Bama Xiang, Baoshan Big-Ear, Luchuan, Min, Jiaxin Black, Mingguang Small-Ear, Wuzhishan, Laiwu, Jinhua, Tibetan pig and Chinese wild boar) were used to genotype the *BMPR1B A746G* mutation. Genomic DNA was amplified with primers M-F (5'-ATTGGAAAAGGTCGCTATGG-3') and M-R (5'-CCAAAATGTTTTCATGCCTCA-3') at an annealing temperature of 59°C. Polymerase chain reaction (PCR) products were visualized in 1.5% agarose gels, and the amplicons were directly sequenced on a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Construction of *BMPR1B* expression vector

Total RNA was extracted from ovaries of healthy Large White sows using the TRIzol Reagent Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA was synthesized using the PrimeScriptRT reagent kit (TakaRa, Shiga, Japan). The synthesized cDNA was used as template to amplify the porcine *BMPR1B* coding region using the gene-specific primers CDS-F1 and CDS-R1 (Table 1), which were designed based on the mRNA sequence of porcine *BMPR1B* gene (GenBank accession no. NM_001039745). To connect the isolated porcine *BMPR1B* CDS with pEF-GFP vector (a gift of Dr. Hongsheng Ouyang at Jilin University), two specific primers, EcoRI-F and NotI-R (Table 1), were designed to amplify the above-mentioned PCR products. The resulting amplicon was then cloned into pMD19-T using the TA cloning kit (TakaRa, Japan) and transformed into *Escherichia coli* (*E. coli*) DH5α competent cells (TakaRa, Japan). The bacteria liquid was used as template for overlap PCR with primers M-F1, M-R1, M-F2, and M-R2 (Table 1) to introduce the *BMPR1B A746G* mutation. The purified PCR product was cloned into pMD19-T (TakaRa, Japan) and transformed into *E. coli* DH5α competent cells (TakaRa, Japan) again. Then the porcine *BMPR1B* CDS with the introduced *A746G* mutation was inserted into the pEF-GFP vector containing human EF1α promoter using T4 DNA polymerase after digestion by *EcoR* I and *Not* I, resulting in the pEF-*BMPR1B* vector. At the final step, PL452 (a gift from Dr. Hongsheng Ouyang at Jilin University) containing *Neo* was digested by *EcoR* I and *Not* I and blunt-ended using *Hind* III and *Xba* I, and pEF-*BMPR1B* was digested by *Hind* III and blunt-ended with T4 DNA polymerase. Both digested fragments of PL452 and pEF-*BMPR1B* were then ligated using T4 DNA polymerase to construct the final vector of 7.7 kb, pEF-*BMPR1B*-Neo (Figure 2A). The thermal cycling conditions of the PCRs for vector construction were all set as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s, 59°C to 61°C (Table 1) for 30 s and 72°C for 1 to 2 min, and followed by a final extension for 5 min at 72°C. To verify the successful introduction of the *BMPR1B A746G* mutation and the integrity of the transgene vector, plasmid DNA was directly

Table 1. Polymerase chain reaction (PCR) primers used in this study

Primer	Sequence (5'-3')	Annealing temperature (°C)	Amplicon (bp)	Enzyme (TaKaRa)
Primers for construction of the vector				
CDS-F1	AAAGGAGCAAGCCTGCCATAC	61	1,607	rTaq
CDS-R1	ACCAACGAGTATCTACCGGCTT			
EcoRI-F	GATTCCAGGAATTCgccacc*ATG*CTT[1]	60	1,544	rTaq
NotI-R	CGATACCGCGGCCGCTCAGAGT*TTA*ATGTC[2]			
M-F1	CCAGAAGCTTGCCACCATG	59	779	rTaq
M-R1	GCCTCATCAACACTGTCGGATAT			
M-F2	GAGACAGAAATATATCGGACAGTG	59	790	rTaq
M-R2	TACCGCGGCCGCTCAGAGTTTAATG			
Primers for characterization of the transgene vector sequence in the host genome[3]				
L1-F	GCCACCTTCTACTCCTCCCCTAGTCAG	68	6240	La Taq
L1-R	AGGGTGGTGGACTTCAGGTAATCATAGAG			
L2-F	GCCACCTTCTACTCCTCCCCTAGTCAG	68	5,475/7,006	La Taq
L2-R	TCCTTTATTAGCCAGAAGTCAGATGCTCA			
N1-F	CCTCGCACACATTCCACAT	58	3,793	La Taq
N1-R	CGCCGCATACACTATTCTCA			
N2-F	TCAAGCCTCAGACAGTGGTTC	62	873	La Taq
N2-R	GTCCCTTTGATGTCTGCAGC			
N3-F	GGCTATTCGGCTATGACTGG	61	821	rTaq
N3-R	GGAGGGGCAAACAACAGAT			
Primers for determination of transgene copy number				
GAPDH-F1	GGCTCTTTCTTTCCTTTCGC	60	369	rTaq
GAPDH-R1	GATGCCTGCTTGCCGAATAGGATGCTCTAGGGTT AGTTTGCT			
NEO-F1	AGCAAACTAACCCTAGAGCATCCTATTCGGCAA GCAGGCATC	60	538	rTaq
NEO-R1	GAGAGGCTATTCGGCTATGAC			
GAPDH-F2	ATGAATGAACCGCCGTTAGG	60	66	Premix ExTaq
GAPDH-R2	AGTCATGGGCAAGTAAGGAGAAAG			
GAPDH-PRO	FAM-AATCTTCCTGAGTCCTTCA-MGB			
NEO-F2	CGGCACTTCGCCCAATAG	60	57	Premix ExTaq
NEO-R2	GTGCTCGACGTTGTCACTGAA			
NEO-PRO	FAM-CCAGTCCCTTCCC-MGB			

[1] Underline indicates *EcoR* I site; italic letters indicate the initiation codon; lower case letters represent Kazark enhancer.
[2] Underline indicates *Not* I site; italic letters indicate the stop codon.
[3] L, long PCR; N, conventional PCR. The L1-F and L2-F primers have the same sequence.

sequenced on the 3130XL Genetic Analyzer (Applied Biosystem, USA) after linearization by *Nhe* I enzyme.

Introduction of the mutated *BMPR1B* gene into porcine fetal fibroblasts

PFF cells were cultured from Large White (USA) fetuses at 30 days of gestation. Cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS), 1% non-essential amino acids, 1% essential amino acids, 1 mM sodium pyruvate, 100 IU/mL penicillin and 100 IU/mL streptomycin at 38.5°C in a humidified atmosphere of 5% CO_2 and 100% air. The *A746G* mutation was first screened for the PFF cells by PCR using primers M-F and M-R, and the PCR products were directly sequenced on the 3130XL Genetic Analyzer (Applied Biosystem, USA). PFF cells were then sex determined by PCR using primers 5'-CCGACGGACAATCATAGC-3' and 5' -GGTGGATGT TACCCTACTGT- 3' with the following conditions: 95°C for 5 min, 30 cycles of (94°C for 20 s, 60°C for 20 s and 72°C for 45 s), followed by a final extension for 5 min at 72°C. The masculine PFF cells were transfected with the pEF-BMPRIB-NEO vector using liposome reagents (FUGene HD; Roche, Basel, Switzerland) at a FUGeneHD (μL) to linearized vector DNA (μg) ratio of 3:1 according to the manufacturer's protocol. Three days after transfection, the cells were cultured in selection medium containing 400 μg/mL of

Geneticin (G418; Sigma, St. Louis, MO, USA) for 2 weeks with medium changed every other day. The G418-resistant colonies were then picked and propagated.

Polymerase chain reaction analysis of transgenic cell lines

Genomic DNA of the picked colonies was extracted using the OMEGA Tissue DNA kit (Bio-Tek, Norcross, GA, USA) according to the manufacturer's instruction. The DNA was used to amplify the vector region spanning the promoter and *BMPR1B* CDS using N2-F and N2-R primers (Table 1). PCR conditions were an initiation of 94°C for 5 min, 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, followed by an extension at 72°C for 10 min.

HMC and culture of reconstructed embryos

Cumulus-oocyte complexes (COCs) were collected from slaughter house-derived sow ovaries and matured in bicarbonate-buffered TCM-199 (GIBCO, USA) supplemented with 10% (v/v) FBS, 10% (v/v) pig follicular fluid, 5 IU/mL human chorionic gonadotropin and 10 IU/mL equine chorionic gonadotropin at 38.5°C in 100% humidified atmosphere of 5% CO_2 for 42 to 44 h.

The HMC was performed as described previously (Vajta et al., 2004; Du et al., 2007; Zhang et al., 2012a). Briefly, cumulus cells were removed from matured COCs using 1 mg/mL hyaluronidase in HEPES-buffered TCM-199. After partial digestion of zona pellucida, oocytes were enucleated by oriented bisection with an ultra-sharp microblade (AB Technology, Pullman, WA, USA). Fusion was performed in two steps. First, each cytoplast without polar body was dropped over a single transgenic fibroblast and fused in fusion medium containing 0.3 M mannitol, 0.1 mM $MgSO_4$ and 0.01% (w/v) polyvinyl alcohol in a 450 fusion chamber (BTX, San Diego, CA, USA) by a single direct current impulse of 2.0 kV/cm for 9 μs; secondly, each cytoplast-somatic cell pair was activated with another cytoplast in specific medium (fusion medium with 0.1 mM $CaCl_2$) using a single direct current impulse of 0.86 kV/cm for 80 μs.

The reconstructed embryos were incubated in porcine zygote medium 3 with the addition of 5 μg/mL cytochalasin B and 10 μg/mL cyclohexinmide for 4 h, and then cultured in PZM-3 supplemented with 4 mg/mL FBS at 38.5°C in 5% CO_2, 5% O_2, 90% N_2, and 100% humidity for 5 to 6 days. About one hundred blastocysts at day 5 or 6 with good morphology were surgically transplanted to each of 8 recipient sows (embryos were transferred into the uterine horns) at 5 or 6 days of standing estrus. Pregnancies were detected by a B-mode ultrasonography machine at 30 days after the surgery.

Polymerase chain reaction screening of transgenic piglets

To check the integrity of the exogenous gene in the cloned transgenic pigs, genomic DNA was extracted from ear tissues of 12 survived boars using standard phenol/chloroform method. DNA quality was detected by a Nanodrop-100 spectrophotometer (Thermo Fisher, Waltham, MA, USA). Fragments of two long PCR and three conventional ones were obtained by amplification of genomic DNA using primers listed in Table 1. DNA of 6 donor cell lines were set for those PCR as well. The amplicons overlapped and covered the entire region of the transgenic vector. PCR products were checked by electrophoresis in 0.8% to 1.5% gels and then directly sequenced on the 3130XL Genetic Analyzer (Applied Biosystem, USA). To distinguish the transgene-positive and negative F_1 pigs, long PCR was performed to amplify the genomic DNA from these individuals using primers L1-F and L1-R (Table 1). Genomic DNA of F_1 offspring was extracted from ear tissues using standard phenol/chloroform method as well.

Genome walking analysis

After PCR analyses of the 12 transgenic pigs, genome walking analysis was conducted to detect the flanking sequence of the exogenous gene of a boar with the highest vector integrity using the GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA). The primers used for the primary and secondary PCR were 5'-TTGCTA GGAGATGCGTATCAGGAGGTA-3' and 5'-AGTAGC GACGAGTGTCTAAGGCAGATG-3', respectively. *Pvu* II restriction enzyme supplied by this kit was chosen to digest genomic DNA based on the vector sequence. The amplified fragments were separated by electrophoresis in 1.5% gels and then directly sequenced on the 3130XL Genetic Analyzer (Applied Biosystem, USA). Further long PCR with primers scanning the transgenic vector and flanking sequence were implemented to validate the insertion sites. The primers for confirming two insertion sites were: SSC12-FP (5'CGCTGTTCTCCTCTTCCTCATCTCC-3') and SSC12-RP (5'-AGGGCATTCTCAGTGTGTCTGT TCGT-3'); SSC13-FP (5'-GCCTTCTATCGCCTTCTTGAC GAGTTCTT-3') and SSC13-RP (5'-GTGCTGCTTCAGG TGTTCCTACTGGATTT-3'), respectively.

Copy number assay of exogenous gene in transgenic animals

Neo and *GAPDH* specific amplicons of 538 bp and 369 bp were generated by conventional PCR with primers NEO-F1/R1 and GADPH-F1/R1 (Table 1), respectively. The two amplicons were linked to form GAPDH-NEO fragment (907 bp) by bridge PCR using primers GAPDH-F1 and NEO-R1 (Table 1). The integrated fragment was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and then sequenced on the 3130XL Genetic Analyzer (Applied Biosystem, USA) to verify the identity.

TaqMan probes and primers (Table 1) were designed for quantitative PCR (qPCR) of target (*Neo*) and reference (*GAPDH*) genes. Both probes were minor groove binder (MGB) ones with carboxyfluorescein (FAM) report label. The amplification of these two genes were detected by absolute quantification. A standard curve was generated using a ten-fold serial dilution of recombinant *GAPDH-NEO* plasmid. qPCR assay was performed for transgenic founder pigs and F_1 offspring produced by No. 11 boar using the premix EX Taq Kit (TakaRa, Japan). The qPCR conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s and 60°C for 1 min. Each sample was analyzed in triplicate. The results were expressed as a fold ratio of the normalized target amounts to the reference amounts.

Analysis of *BMPR1B* expression in F_1 piglets

Boar No. 11, which had the entire exogenous promoter and CDS, was chosen to cross with non-transgenic Large White sows (Canada). Two transgene-positive and two negative F_1 female siblings were slaughtered at 3 days of age to collect tissues for expression analyses of the *BMPR1B* gene.

Total RNA was extracted using the TRIzol Reagent kit (Invitrogen, USA), and cDNA was synthesized using the PrimeScriptRT reagent kit (TaKaRa, Japan). The cDNA templates were used to amplify the *A746G* mutation with primers M-F and M-R (Table 1). The PCR condition was the same as that for the vector construction. PCR products were cloned to pGEM-T vector (Promega, USA) and then used for blue-white colony screening. PCR products were directly sequenced on the 3130XL Genetic Analyzer (Applied Biosystem, USA).

Total protein was extracted from tissues using a Total Protein Extraction Kit (Applygen, Beijing, China). Approximate 40 μg protein of each sample was loaded on a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The samples were run at 80 V for 20 min in stacking gels (5%) and at 120 V for 40 min in resolving gels (8%). The separated polypeptides were transferred from the slab gels to 0.45 μm polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) at 35 mA for 3 h in transfer buffer (38.63 mM glycine, 250 mM Tris, 1 g/L SDS, 20% methanol). The blotted membrane was blocked with 5% skim milk in tris-buffered saline with Tween 20 (TBST) (150 mM NaCl, 10 M Tris-HCl pH 8.0, 1% Tween-20) for overnight at 4°C. After washing the membrane with TBST for 3 times, BMPR1B antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBST (1:5,000) was added and incubated for 3 h at room temperature. The bound antibodies were detected using horseradish peroxidase conjugated goat anti-rabbit immunoglobulins (ZSGB-BIO, Beijing, China) at dilution 1:5,000 in TBST as secondary antibody. Beta actin was used as loading control. The anti-beta actin antibody (Abcam, Cambridge, UK) and Peroxidase-Conjugates AffiniPure Goat Anti-Mouse IgG (H+L) (ZSGB-BIO, China) were used as primary and secondary antibodies accordingly. The bands were visualized with the ECL Chemiluminescent kit (Transgen, Beijing, China) and scanned with GeneSnap software on a GeneGnome Scanner (Syngene, Cambridge, UK). Band intensities were quantified using GeneTools software (Syngene, UK). The relative amount of BMPR1B was calculated after correction for the amount of β-actin.

Reproductive trait analyses

Boar No. 11 was mated to 7 wild-type Large White (Canada) sows to produce 1 to 2 litters each. Three transgene-positive F_1 boars were further selected to cross with F_1 sows. F_1 sows from the same litter, either positive or negative, were mated with the same boar avoiding full-sib mating. Reproductive performances, including the total number of piglets born (TNB), the number of piglets born alive (NBA), the number of weak piglets (NW), the number of mummies (NM) and the number of still-born (NSB), were recorded.

Semen collection and quality assessment of transgene-positive and negative F_1 boars were performed as described previously (Xing et al., 2009). Briefly, boars were trained 15 to 30 min per day to mount and mate dummy sow from 280 days of age. After two successful matings, semen of each boar was collected for quality assessment by glove-hand method 4 times at an interval of 4 days. Sperm motility was detected with a light microscope at 200×magnification immediately after semen collection. Semen volume of each ejaculation was determined using a graduated cylinder. Sperm concentration was measured with haemocytometer slides after diluting semen samples with 3% NaCl solution. The total sperm number per ejaculate was calculated by multiplying the semen volume by the sperm concentration. Six transgene-positive and 6 negative boars from three litters with good health conditions were selected for semen collection blind-labeled. The R language was used to perform the t-test statistical analysis.

RESULTS

Detection of the *BMPR1B A746G* mutation and the transgene vector in swine and porcine fetal fibroblasts

In this study, the *G* allele of the *BMPR1B A746G* mutation site was absent in all tested animals (n = 280) representing 3 Western commercial breeds and 11 Chinese local breeds. All individuals were *AA* homozygotes. PFF

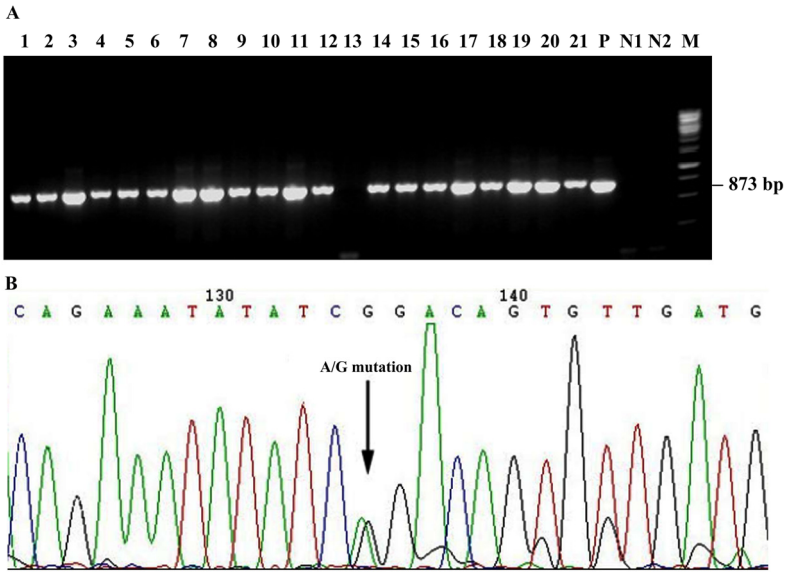

Figure 1. Identification of transgenic donor cells. (A) Detection of the transgenic cells. Lanes 1 to 21 indicate the amplification results of different geneticin-resistant cell clones. P, transgenic plasmid DNA; N1, water; N2, untransfected porcine fetal fibroblasts. M indicates 1 kb DNA Ladder. (B) Sequencing result from polymerase chain reaction product of one transgenic donor cell line. The arrow indicates the introduced mutation site.

cells for transfection were also screened and *GG* and *GA* genotypes were not found. Direct sequencing analysis showed that the transgene vector has a size of 7.7 kb containing the 1,509 bp *BMPR1B* CDS with the introduced *G* allele and complete sequence of promoter, rabbit globin polyA terminator and *neo* regions.

Screening of the transgenic cells

A total of 91 clones were picked and genotyped via PCR-sequencing. Promoter and CDS were successfully amplified in 73 of the 91 clones (data not shown), indicating that the majority of G418 geneticin-resistant cell clones were transgenic (Figure 1A). In addition, we confirmed the existence of the induced *BMPR1B A746G* mutation in the host genome by sequencing of the clones (Figure 1B).

Transgenic pigs produced by handmade cloning

Thirty days after the embryo transfer, 7 out of 8 recipients were confirmed to be pregnant with B-mode ultrasonographic examination. A total of 24 piglets were obtained from 5 recipient sows after 112 to 116 days gestation (Table 2). One of these recipients gave birth by Cesarean section and four piglets were born dead. Of these 20 naturally born piglets, 12 survived to maturity, and the others died mostly of diarrhea before weaning. The birth weight of the 24 cloned transgenic piglets ranged from 0.60 to 1.50 kg, with a mean of 0.99 kg. The average weight of the surviving 12 piglets was 1.03 kg in average, ranging from 0.70 to 1.50 kg.

Identification of cloned transgenic pigs

To detect the integrity of the exogenous transgenic vector in the 12 matured cloned pigs, we performed two

Table 2. Production of cloned transgenic pigs

Recipient sows ID	No. of transferred blastocysts	Pregnancy	Cell lines[1]	Gestation (d)	Born piglets	Number born alive	Boars till maturation
1	94	Yes (Abortion)	C1	-	-	-	-
2	84	Yes	C3	116	4	4	No.11
3	94	Yes (Abortion)	C3	-	-	-	-
4	84	Yes	C1	110	2	2	No.1, 2
5	121	No	C1/C4	-	-	-	-
6	110	Yes	C1/C2/C3/ C4/C6	112	8	8	No.6, 7, 8, 9, 10, 12
7	87	Yes	C1/C4/C5	113	6	6	No.3, 4, 5
8	104	Yes	C1/C2/C4	114	4[2]	0	-

[1] These cell lines were selected from 91 clones.
[2] The four piglets were obtained by Caesarean section and all of them were dead at birth.

long-PCR using the primers spanning the majority of the vector. The result of first long-PCR indicated that only No. 11 had the vector spanning the beginning of vector and the majority of the CDS (Figure 2B). The second long-PCR using primers spanning the whole vector showed that only No. 12 had a fragment, smaller in size than the expected length (Figure 2C). Then we implemented two conventional PCRs, one spanned the front part of the vector (Figure 2D) and the other spanned the pivotal promoter and CDS regions (Figure 2E). The former PCR showed that all except No. 6 were transgene-positive (Figure 2D), and the latter PCR (Figure 2E) together with the long PCRs (Figure 2B and 2C) revealed that only No. 11 had the full promoter and CDS regions but lacked part of the terminator. We further designed an extra pair of primers spanning the *Neo* region, and the PCR confirmed that No. 6 was transgene-negative (Figure 2F). In the above-mentioned five PCRs, DNA of 6 donor cell lines served as control. All had the expected

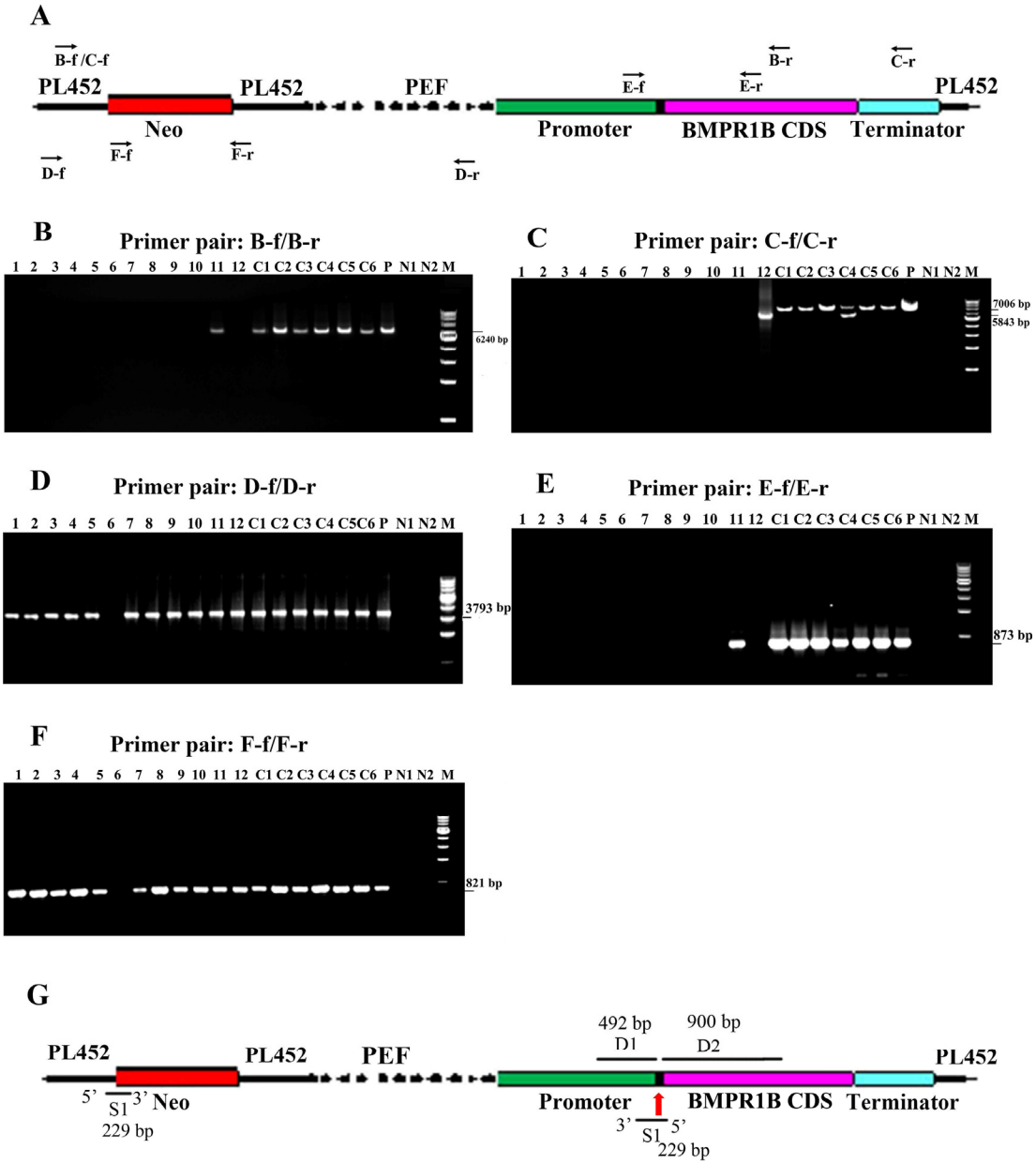

Figure 2. Polymerase chain reaction (PCR) detection of cloned transgenic pigs. (A) Diagrammatic representation of the pEF-*BMPR1B*-*Neo* transgenic vector and PCR primer design. B, C, D, E, and F indicate positions of primers. Primers B, C, D, E, and F are identical to the L1, L2, N1, N2, and N3 in Table 2. The f and r denote the primer orientations (forward and reverse). PL452 and PEF indicate the backbones of pEF-GFP and PL452 vectors used in this study. (B-F) PCR analyses of cloned transgenic pigs and donor cell lines. Lanes 1-12, PCR products of the 12 cloned transgenic pigs; C1-C6, PCR products of donor cell lines; P, N1, and N2 represent the transgenic plasmid DNA, water and DNA of non-transgenic pig, respectively. M, 1 kb DNA Ladder. (G) Sequence analysis of PCR products from pig No. 12 and 5,843 bp fragment of C4 cell line in (C). D, deletion; S1, insertion fragment. The arrow indicates the insertion site.

fragments of right sizes (Figure 2B to 2F). An extra band showed up in C4 cell line in PCR, and it had the same size as that for pig No. 12 (Figure 2C). To verify the identity of exogenous vector in those 12 pigs and 6 donor cell lines, all PCR products were purified and directly sequenced. The data (not shown) indicated that the smaller fragment (5,843 bp) from C4 had the same sequence as that of No. 12. All other fragments were identical to the plasmid DNA. Sequencing data for No. 12 revealed that 2 deletions and 1 conversed insertion happened to the transgenic vector in these two products. The two deleted segments were located in the promoter and the CDS, with fragment sizes of 492 bp and 900 bp, respectively. The inserted segment of 229 bp was located between the promoter and the CDS (Figure 2G). Combining the PCRs and sequencing results, we concluded that all except one (No. 6) cloned pigs were transgene-positive. However, none of the 11 transgene-positive pigs had the intact exogenous vector, and 10 of them lost part or whole promoter and CDS regions. With regard to donor cell lines, the C4 cell line contained at least two clone populations, one had an intact transgene cassette and the other carried rearrangements.

Transgenic insertion site of pig No. 11

By applying genome walking analysis, we detected two insertion sites for the transgenic vector in No. 11 pig (Figure 3). One insertion site was located within exon 6 of the *NAGLU* gene at 20,485,379 bp on pig chromosome (SSC) 12 (*Sscrofa* 10.2) (Figure 3A). The other site was located within intron 1 of the *CMTM8* gene at 20,503,293 bp of SSC 13 (Figure 3B). Further PCR spanning the beginning of vector and the 3' flanking sequence verified these two insertion sites, and the fragment sequences showed that the full vector sequences were inserted into the host genome (Figure 3C) except the lost part of the terminator sequence (Figure 3A and 3B).

Detection of the copy numbers of the exogenous gene

qPCR revealed that all founder pigs except for pig No. 6 had 1 to 2 copies of the *Neo* gene (Figure 4A), confirming that pig No. 6 was a transgenic negative individual. Of the 11 transgene-positive pigs, No. 4, 9, and 12 had one copy and the rest had two copies of *Neo*. The copy number of pig No. 11 (two copies) was concordant with the genome walking analysis of this individual. In addition, the F_1 pigs

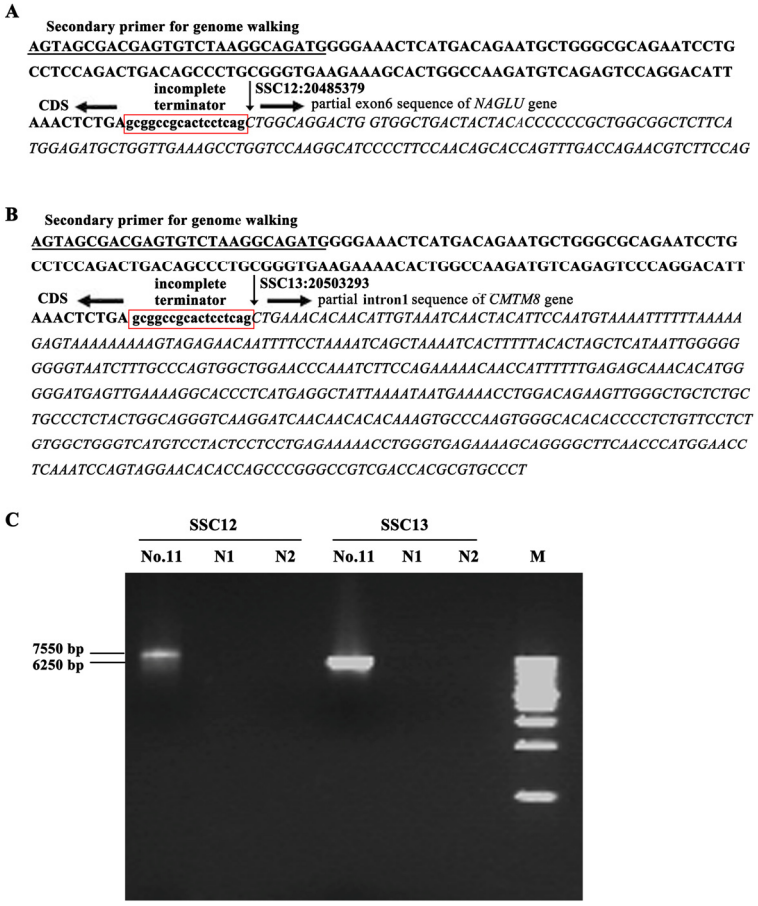

Figure 3. The integration sites of the transgenic vector of No. 11 boar. (A) The integration site on SSC12. (B) The integration site on SSC13. The vertical arrows indicate the insertion site. (C) Polymerase chain reaction verification of two integration sites of pig No. 11. N1 and N2 represent the DNA of non-transgenic pig and water, respectively. M indicates 1 kb DNA Ladder Marker.

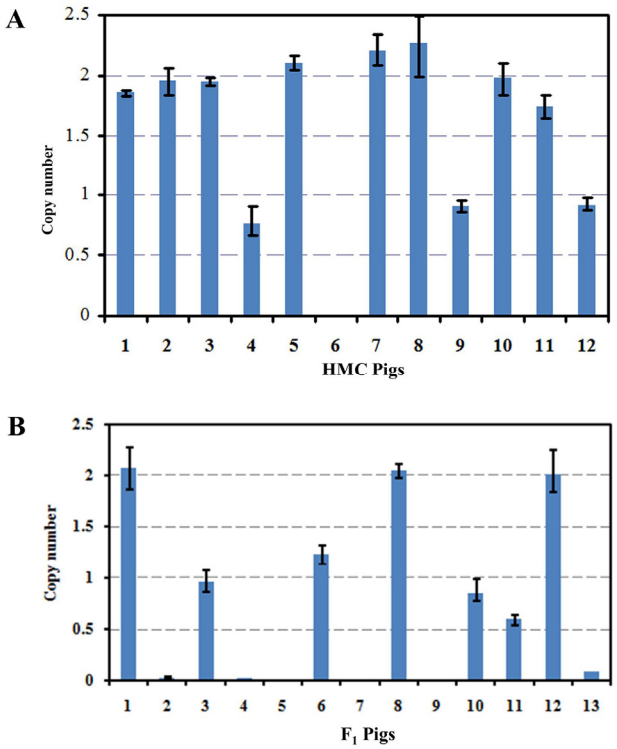

Figure 4. Copy number of exogenous *Neo* in transgenic pigs. (A) 12 cloned transgenic pigs and (B) a litter of F_1 pigs produced by No. 11 founder boar determined by quantitative polymerase chain reaction. Standard deviation bars are shown.

generated from No. 11 boar had 0, 1, or 2 copy numbers of the exogenous gene (Figure 4B).

Detection of the expression of the *BMPR1B* gene

Blue-white screening of cDNA of F_1 positive piglets revealed that the introduced mutant allele (*746G*) was expressed in brain, cerebellum, liver, lung, spleen, small intestine, skeletal muscle and ovary but not in stomach and bladder. In comparison, the wild-type allele (*746A*) was found in all 10 tissues (Figure S1).

Western blotting results showed that the BMPR1B protein expression levels in the spleen, muscle, kidney, lung, liver and small intestine samples of transgene-positive piglets were 1.25, 1, 1, 0.5, 1, and 2 times higher than those of transgenic negative piglets (Figure 5).

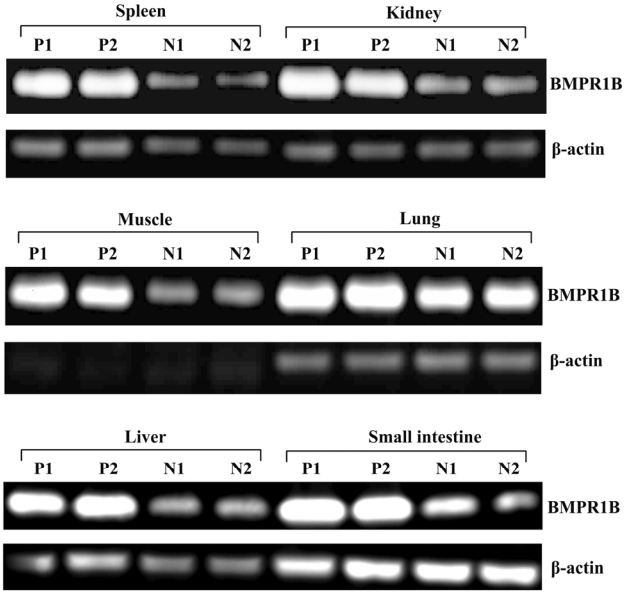

Figure 5. Western blot analysis of BMPR1B in multiple tissues from F_1 piglets. P1 and P2, transgene-positive pigs, N1 and N2, transgene-negative pigs.

Reproductive performance of transgenic pigs

In this study, boar No. 11 was mated with 7 wild-type Large White sows and gave birth to 13 litters of F_1 pigs. These sows had an average of 12.77 TNB, 11.85 NBA, 0.77 NW, 0.31 NM, and 0.62 NSB, respectively (Table 3). Transgene-positive F_1 boars were selected to cross with transgene-positive and negative sows. The positive and negative F_1 sows had average 11.22 and 12.10 TBN, 9.11 and 10.20 NBA, 0.33 and 0.20 NW, 0.11, and 0.10 NM and 2.00 and 1.80 NSB, respectively.

Regarding semen characteristics, transgene-positive boars had higher semen volume, sperm concentration and total sperm per ejaculate than negative ones, but the

Table 3. Litter size performance of transgenic pigs

Traits	F_0 (N = 13)		F_1			
			Transgene-positive[1] (N = 9)		Transgene-negative (N = 10)	
	Mean±SE	Range	Mean±SE	Range	Mean±SE	Range
TNB	12.77±0.85[a]	10-22	11.22±1.01[a]	5-15	12.10±0.90[a]	6-15
NBA	11.85±0.77[a]	9-20	9.11±1.03[a]	3-10	10.20±1.01[a]	4-14
NW	0.77±0.28[a]	0-1	0.33±0.24[a]	0-2	0.20±0.13[a]	0-1
NM	0.31±0.17[a]	0-2	0.11±0.11[a]	0-2	0.10±0.10[a]	0-1
NSB	0.62±0.31[a]	0-4	2.00±0.33[b]	0-4	1.80±0.65[b]	0-4

F_0, litter size performance of No. 11 boar and wild-type Large White sows; F_1, litter size performance of F_1 transgene-positive boars and F_1 sows; N, number of litters; SE, standard error; TNB, the total number of piglets born; NBA, the number of piglets born alive; NW, the number of weak piglets; NM, the number of mummies; NSB, the number of still-born.

[1] Copy number detection revealed that 6 of 9 transgene-positive sows had both integration sites of exogenous vector (data not shown).

Different small letters in the same row indicate significant difference at 0.05 level.

differences were not significant (Table 4). The most significant difference was found in semen volume (322.59 vs 288.55, p = 0.06). These two boar groups had same mean value of sperm motility (Table 4).

DISCUSSION

In this study, we generated 24 cloned transgenic piglets by 5 recipient sows using the HMC technique (Table 2) and 12 survived until maturation. This work had a similar cloning efficiency compared with the previous study (Zhang et al., 2012a), who obtained 16 piglets from three recipients. Our study illustrated that the HMC technique is robust for the generation of cloned transgenic animals.

To detect the integrity of the exogenous transgenic vector in the 12 pigs that reached sexual maturity, we conducted a series of PCR analyses and found that only No. 11 had the entire promoter and the CDS regions. We then performed genome walking to detect the flanking sequences of the transgene cassette in No. 11 boar. Two integration sites were found, and both had the full promoter and CDS sequence and shortened terminator (Figure 3A, 3B, and 3C). This well explained that No. 11 had amplification products in the first long PCR (Figure 2B) but not in the second long PCR (Figure 2C) when detecting transgenic vector integrity. Further, we implemented absolute quantification analysis using primers spanning the *NEO* region that existed in all transgene-positive pigs (Figure 2D and 2F). The results confirmed that No. 6 and No. 11 had 0 and 2 copies of the exogenous vector, respectively (Figure 4A). Moreover, the copy numbers of *Neo* in F_1 pigs (0, 1, or 2) produced by No. 11 boar showed the stable inheritance of the exogenous gene (Figure 4A and 4B). High frequencies of transgenic aberrations such as deletion, duplication and rearrangement have been found in transgenic animals produced by microinjection (Mark et al., 1992; Chen et al., 1995; Nakanishi et al., 2002; Forsberg et al., 2013). In contrast, the transgene rearrangement seems to be greatly reduced either in the donor cells grown in culture or in the transgenic animals produced by SCNT (Atala et al., 2011). In our study, we selected donor cells under G418 and isolated clones that were identified positive by PCR using primers spanning promoter and CDS (Figure 1A). In PCR screening of 12 cloned transgenic pigs and 6 donor cell lines, C4 donor cell line was later found to be a mixed population. The other 5 cell lines showed expected unique PCR products. However, none of the transgene-positive pigs had the full transgenic vector, suggesting that the vector was disrupted during cloning, whereas the causes were unknown.

Human EF1α promoter is a very strong and non-tissue specific promoter. Therefore, human EF1α-drived transgene (the mutant *BMPR1B*) is expected to be highly expressed in multiple tissues. Indeed, we found the expression of the introduced mutant allele (*746G*) in 8 tissues (Supplementary Figure S1). Moreover, the western blot data showed that the protein expression levels in all detected tissues were higher in transgene-positive individuals (Supplementary Figure S2) than in negative ones (Figure 5). The overexpression of the mutant *BMPR1B* in multiple tissues could have undesirable effects on transgenic positive individuals. Nevertheless, those transgenic pigs showed comparable growth and immune traits with negative ones (data not shown).

As only pig No. 11 contained the complete promoter and CDS regions of the transgenic vector, we selected it to cross with wild-type Large White sows to produce F_1 pigs. The litter size data indicated that the No. 11 founder boar had good litter size performance, averaging 12.77 and 11.85 of TNB and NBA (Table 3), respectively. Positive and negative F_1 sows had lower TNB and NBA (positive: 11.22 TNB and 9.11 NBA; negative: 12.10 TNB and 10.20 NBA; Table 3) and higher NSB than the F_0 group. This may be due to the half-sib mating of the F_1 groups or small sample size. The F_1 positive sows had slightly lower TNB and NBA, as well as slightly higher number of NW, NM, and NSB than the negative ones, but not statistically different. We deduce that this was possibly due to the disruption of N-acetylglucosaminidase (*NAGLU*) gene by the integration site on SSC12 or small sample size. *NAGLU* has been implicated in Sanfilippo syndrome disease in several mammals including human (Verhoeven et al., 2010), mouse (Langford-Smith et al., 2011), dog (Ellinwood et al., 2003) and cattle (Karageorgos et al., 2007). However, no porcine models for this disease have been reported. Detailed biological effects of disrupting this gene in porcine should be further investigated. Another integration site on SSC13 was located within the large intron 1 (97.5 kb) of *CMTM8*, a potential tumor suppressor gene (Zhang et al., 2012b). Sow litter size has been widely regarded as a complex trait affected by various elements including physiological, genetic and environmental factors and their interactions

Table 4. Semen characteristics of transgene-positive and negative F_1 boars (Mean±standard error)

	Semen volume (mL)	Sperm motility	Sperm concentration (million/mL)	Total sperm per ejaculate (109)
Positive (N = 6)	323.59±12.57	0.87±0.02	232.34±21.85	73.36±7.30
Negative (N=6)	288.55±12.79	0.87±0.01	225.21±12.41	62.61±2.55
p value	0.06	0.87	0.78	0.18

(Urban et al., 1966; Leymaster and Bennett, 1994; Humpolicek et al., 2012), thus large numbers of litters are required to accurately estimate the litter size performance between transgene-positive and negative sows. In addition, as introns are considered a safer location to contain exogenous genes (Renault and DuChateau, 2013; Awe et al., 2014), we should further identify the transgene-positive sows with only a SSC13 integration site for comparing litter size performance with transgene-negative sibling sows.

It has been shown that Garole×Malpura rams with *BMPR1B A746G* genotype have higher sperm concentration than those with wild genotype (Kumar et al., 2007). In this study, we compared routine semen characteristics including semen volume, sperm motility, sperm concentration and total sperm per ejaculate between full-sib F_1 positive and negative boars. To accurately compare the semen characteristic data, 6 positive and 6 negative boars with good health conditions were chosen from three full-sib litters. In addition, all boars were blind-labeled to the experimenters. Semen was collected in the same period for all boars by the same personnel. The data showed that the positive ones had the same mean value of sperm motility as the negative individuals. Interestingly, the positive boars had higher semen volume, sperm concentration and total sperm per ejaculate than the negative ones, but with no significant difference (Table 4). This phenotype is similar to the findings in rams reported by Kumar et al. (Kumar et al., 2007). Although most function studies for bone morphogenetic protein genes were focused on female reproduction traits, there were a few reports indicating that these genes also play important roles on male reproduction traits (Shimasaki et al., 2004). Our study provides new proof that *BMPR1B* may be involved in important functions of male reproduction in mammals.

To our knowledge, there are no reports on litter size and semen quality performance of cloned transgenic pigs. It has been reported that Large White sows in Canada had an average of 13.4 TNB and 11.5 NBA at the first parity (2014 Annual Report of Canada Center for Swine Improvement, https://www.ccsi.ca/meetings/annual/Annualreport_2014_EN.pdf). In our study, Large White sows from Canada that mated with the transgenic founder boar showed similar litter size performance (12.77 TNB and 11.85 NBA for the first to second litters). In addition, the F_1 positive and negative sows also showed good TNB performance (11.22 for positive and 12.10 for negative sows, Table 3), though they were inseminated by half-sib mating. The sperm production between different studies are variable in commercial Large White boars. For example, Czech and Polish Large White boars have an average of 101.3×10^9 total sperm per ejaculate (Wolf and Smital, 2009) and Polish Large White boars have 82.93×10^9 (Knecht et al., 2014). In this study, the transgene-positive F_1 boars had an average of 73.36×10^9 of total sperm per ejaculate and negative ones had 62.61×10^9, both lower than the reported data from commercial wild-type Large White boars. This may be due to the breeding, management and climatic differences among different breeds.

In this study, we successfully introduced a missense mutation related to sheep prolificacy into porcine genome, expecting the host sows to exhibit better litter size performance. However, the positive sows showed slightly poorer litter size traits than the negative ones. This may be partly due to the unfavorable insertion site of the exogenous vector. In recent years, CRISPR/Cas9-mediated genome engineering technology has been widely used by scientists all over the world because of its high precision and efficiency of genome editing. This system may be a useful tool to precisely introduce point mutations into porcine genome.

CONCLUSION

In conclusion, we obtained 24 cloned transgenic pigs with the modified porcine *BMPR1B* CDS using HMC. Only one surviving boar (No. 11) had the full promoter and the *BMPR1B* CDS regions, and the transgenic vector was most likely disrupted during the cloning. cDNA sequencing and Western blot indicated that the exogenous *BMPR1B* CDS was successfully expressed in host pigs. No. 11 founder boar had normal litter size performance. Unexpectedly, transgenic positive F_1 sows showed slightly poorer litter size performance than the negative ones, probably due to the disruption of a functionally important gene or the small sample size. F_1 positive boars showed better sperm producing ability than negative individuals, but with no statistical significance. Our study provides new insights into production of cloned transgenic pigs related to reproductive traits.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from National Key Research Program of China (2014ZX08006-005) and Science and Technology Supporting Program of Jiangxi Province (20121BBF60030).

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