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Transgenic Efficiency of FoxN1-targeted Pig Parthenogenetic Embryos

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ABSTRACT

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein (Cas9) system can be applied to produce transgenic pigs. Therefore, we applied CRISPR/Cas9 system to generate FoxN1-targeted pig parthenogenetic embryos. Using single guided RNA targeted to pig FoxN1 genes was injected into cytoplasm of in vitro matured oocyte before electrical activation. In results, regardless of the concentrations of vector, the cleavage rate were significantly (p<0.05) decreased (4 ng/μl, 51.24%; 8 ng/μl, 40.88%; and 16 ng/μl; 45.22%) compared to no injection group (70.44%). The blastocyst formation rates were also decreased in vector injected 3 groups (4 ng/μl, 7.96%; 8 ng/μl, 6.4%; and 16 ng/μl; 9.04%) compared to no injection group (29.07%). In addition, the blastocyst formation rates between sham injected group (13.51%) and no injection group (29.07%) also showed significant difference (p<0.05). The mutation rates were comparable between groups (4 ng/μl, 18.4%; 8 ng/μl, 12.5%; and 16 ng/μl; 20.0%). The sequencing analysis showed that blastocysts derived from each group were successfully mutated in FoxN1 loci regardless of the vector concentrations. However, the deletion patterns were higher than the patterns of point mutation and insertion regardless of the vector concentrations. In conclusion, we described that cytoplasmic microinjection of FoxN1-targeted CRISPR/Cas9 vector could efficiently generate transgenic pig parthenogenetic embryos in one-step.

(Key words : CRISPR/Cas9 system, FoxN1, pig, parthenogenetic embryos)

INTRODUCTION

Transgenic pigs are very important for the research of agricultural and medical science (Whyte and Prather, 2011). However, to produce transgenic pigs, huge endeavor are required from human to material resources because of its complicated and difficult procedures. Regardless of its difficulties, the first transgenic pig was produced about 3 decades ago by a method of pronuclear microinjection (Hammer *et al.*, 1985). Thereafter, another reliable method for production of transgenic pig was applied using gene-targeted somatic cells such as somatic cell nuclear transfer (Lai *et al.*, 2002). The pronuclear microinjection and somatic cell nuclear transfer methods are having weakness in the efficiency of productivity and its complicated procedures, respectively. Therefore, some meganucleases such as zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated protein (Cas9) system (CRISPR/Cas9 system) were developed to improve and simplify the efficiency and procedure. The ZFNs, TALENs, and CRISPR/Cas9 system showed advanced efficiency and simplified procedure (Cong *et al.*, 2013; Gaj *et al.*, 2013; Hauschild *et al.*, 2011). Additionally, the CRISPR/Cas9 system was reported more recently than others with successful production of transgenic animals including pig (Hai *et al.*, 2014), monkey (Niu *et al.*, 2014), mouse (Wang *et al.*, 2013), rat (Li *et al.*, 2013), drosophila (Yu *et al.*, 2013) and zebra fish (Hwang *et al.*, 2013).

The present study was designed to investigate whether cytoplasmic injection of CRISPR/Cas9 vector can generate the FoxN1-targeted pig parthenogenetic embryos. Additionally, the targeting efficiency and developmental competence were inves-

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tigated within different concentrations of CRISPR/Cas9 vector.

MATERIALS AND METHODS

1. *In Vitro* Maturation

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise. Briefly, prepubertal pig ovaries were obtained from gilts at a local slaughterhouse and transported to the laboratory in saline at around 30 to 35℃ within 1 h. Cumulus-oocyte complexes (COCs) were aspirated from follicles (3～6mm diameter) by 18 gauge needle attached to a 10 ml disposable syringe. The follicular fluid with COCs were collected into conical tubes and washed 3 times in tissue culture medium (TCM)-199 containing 0.1% (w/v) polyvinyl alcohol (PVA). After sedimentation, the COCs with several layers of compact cumulus cells were selected for in vitro maturation (IVM). After selection, about 50～70 COCs were transferred into 500 μl of TCM-199 medium (Gibco BRL, USA) supplemented 10% porcine follicular fluid (pFF), 3.05 mM D-glucose, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 0.5 μg/ml FSH, 0.5 μg/ml LH, 10 ng/ml EGF, 75 μg/ml penicillin G and 50 μg/ml streptomycin in a four-well dish. The COCs were matured for 22 h with hormone and 22 h without hormone at 39℃ under 5% CO_2 in air.

2. Construction of CRISPR/Cas9-Mediated Exogenous Vector

The genomic DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen, Mannheim, Germany) following manufacturer's protocol. The region of DNA containing the engineered nuclease target site was amplified by PCR with primer forward 5′-TCCAGCTCTCACCCTTGGAC-3′ and reverse 5′-TCCGACCCCAGGATTTGGGG-3′. The amplicons were denatured by heating and annealed to form heteroduplex DNA, which was treated with 5 units of T7 endonuclease 1 (New England Biolabs, Beverly, MA, USA) for 1 h at 37℃ and then analyzed by 2% agarose gel electrophoresis. Then, five guide RNAs (gRNAs) has designed specific to exon 2 in the FoxN1 gene and cloned pX330 vector by addgene (Addgene, Cambridge, MA, USA). To confirm the *in vitro* DNA cleavage activities of CRISPR/Cas systems, composition of synthetic gRNAs and recombinant Cas9 protein expression in porcine ear fibroblast (pEF) was examined.

3. Confirmation of Mutant Frequency by Sequencing

The genomic DNA isolated from Cas9 targeted oocytes was amplified by PCR with primer forward 5′-TCCAGCTCTCACCCTTGGAC-3′ and reverse 5′-TCCGACCCCAGGATTTGGGG-3′, for the region spanning exon 2. These PCR products were sub-cloned using a TA Cloning Kit (Promega, Madison, WI, USA) following manufacturer's protocol. Sub-cloned amplicons were sequenced with primers used for PCR amplification.

4. Cytoplasmic Injection of Exogenous Vector

After IVM, cumulus cells were removed from oocytes by gentle pipetting after incubating the COCs in PBS supplemented with 0.1% hyaluronidase and 0.1% PVA for 5 min. The exogenous vector were injected into the oocytes with visible first polar body within TCM 199 medium supplemented with 0.59 mM sodium bicarbonate, 3.14 mM HEPES, 30.2 mM sodium chloride, 50 μg/ml penicillin G, 60 μg/ml streptomycin, 5 μg/ml cytochalasin B. Microinjection was carried out using an inverted microscope (Olympus, Tokyo, Japan) equipped with a micromanipulating arms (Narishige, Tokyo, Japan). The CRISPR/Cas9 vector were diluted in Tris-EDTA buffer (TE buffer) and injected with different concentrations of 0 (sham injection), 4, 8 and 16 ng/μl.

5. Parthenogenetic Activation and *In Vitro* Culture

Microinjected oocytes were activated in medium containing 0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 mM HEPES. In between 0.2 mm diameter of electrodes, 2 direct current pulses (1 sec interval) of 1.25 kV/cm were applied for 30 μsec using an Electro Cell Fusion (NEPA gene, Chiba, Japan). Activated embryos were washed and cultured in porcine zygote medium-3 within four-well dish at 39℃ under 5% CO_2 in air. During 7 days of culture period, the embryos were examined the cleavage and blastocyst formation rates.

6. Statistics

Statistical analysis was carried out by statistical analysis system software (SAS software, SAS Institute, Cary, NC, USA). The average of cleavage rate and blastocyst formation rate were determined by Duncan's multiple range tests. All data were showed as mean ± SEM (Standard Error of the sample Mean). A probability at $P<0.05$ was considered significant.

RESULTS

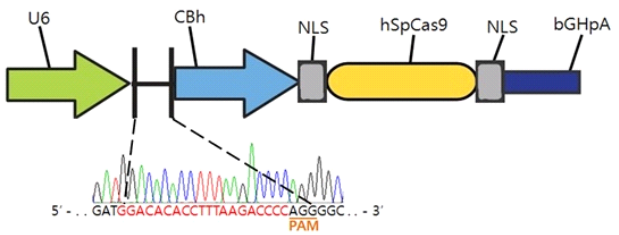
1. Design and Construction of CRISPR/Cas9-mediated Vector

As shown Fig. 1 (A), five CRISPR/Cas9 sites were designed for targeting Fox*N1* exon 2 on chromosome 12 with PAM sequences for recognizing by Cas9 protein. Then, these five gRNAs have cloned in pX330 vector by Addgene (Cong *et al.*, 2013). These five gRNAs were transfected into porcine ear fibroblast genomic DNA with CRISPR/Cas9 vector containing different target gRNAs to select optimal heterologous action. In result, foxnt4 (5'-GGACACACCTTTAAGACCCC-3') was selected for the next experiment because of its optimal cleavage form (2 bands) and size (150～200 bp) by T7E1 assays. These five single gRNAs were driven by the U6 promoter and humanized Streptococcus Pyogenes Cas9 (hSpCas9) driven by hybrid chicken β-actin (CBh) promoter (Fig. 1 (B)).

2. Developmental Competence of CRISPR/Cas9-mediated Vector Injected Pig Parthenotes

As shown in Table 1, regardless of the concentrations of vector, the cleavage rate were significantly (p<0.05) decreased (4 ng/μl, 51.24%; 8 ng/μl, 40.88% and 16 ng/μl; 45.22%) compared to no injection group (70.44%). However, the cleavage rate between 3 groups of vector injected and sham injection group (55.4%) showed no difference. The blastocyst formation rates were also decreased in vector injected 3 groups (4 ng/μl, 7.96%; 8 ng/μl, 6.4% and 16 ng/μl; 9.04%) compared to no injection group (29.07%). In addition, the blastocyst formation rates between sham injected group (13.51%) and no injection group (29.07%) also showed significant difference (p<0.05).

(A)



(B)

Fig. 1. Primers and target site of *Sus scrofa* FoxN1 sequence (A) and CRISPR/Cas9-mediated vector construction (B). Five CRISPR/Cas9 target sites and PAM sequences (NGG) are highlighted as green and yellow, respectively (foxnt1 to foxnt5) within red highlights of FoxN1 primers. Target sites has designed specific to exon 2 in the *FoxN1* gene and cloned pX330 vector by addgene.

Table 1. Developmental competence of CRISPR/Cas9-mediated vector injected pig parthenotes

Vector concentrations	No. of oocyted injected	No. (%)* of embryos developed to	
		2-cell	Blastocyst
4 ng/μl	201	103 (51.24 ± 4.2)^{b}	16 (7.96 ± 2.8)^{b}
8 ng/μl	203	83 (40.88 ± 6.8)^{b}	13 (6.40 ± 1.8)^{b}
16 ng/μl	199	90 (45.22 ± 5.4)^{b}	18 (9.04 ± 2.5)^{b}
No injection	172	121 (70.44 ± 2.4)^{a}	50 (29.07 ± 5.9)^{a}
Sham Injection	74	41 (55.40 ± 1.7)^{ab}	10 (13.51 ± 2.0)^{b}

Groups of no injection (Electrical activation only) and sham injection (TE buffer only) were used as control groups for injection and vector, respectively.

* Percentages are expressed as mean ± SEM of seven replicates.

^{a,b} Different superscripts denote significant differences with in columns (p<0.05).

3. Knockout Efficiencies and Mutation Patterns

Overall 33 blastocysts from the vector injected groups, 350 colonies were picked up and the sequencing analysis was carried out. As shown in Table 2, the mutation rates were comparable between groups (4 ng/μl, 18.4%; 8 ng/μl, 12.5% and 16 ng/μl; 20.0%). The sequencing analysis showed that blastocysts derived from each group were successfully mutated in FoxN1 loci regardless of the vector concentrations (Fig. 2 (A)). However, the deletion patterns were higher than the patterns of point mutation and insertion regardless of the vector concentrations (Fig. 2 (B)).

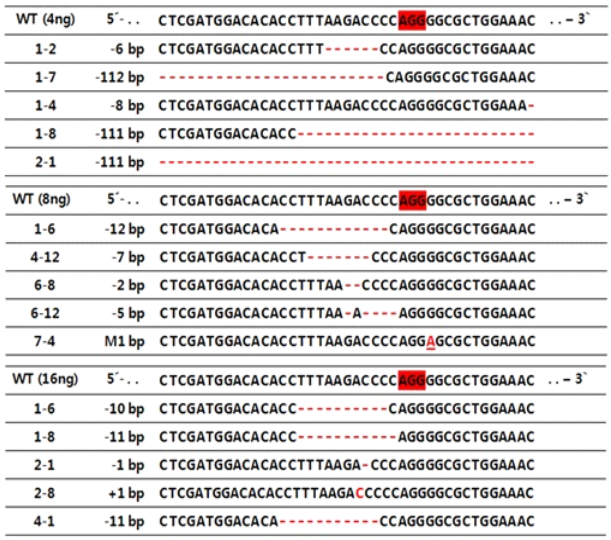
DISCUSSION

In pigs, utilization of gene targeting skill to produce a trans-

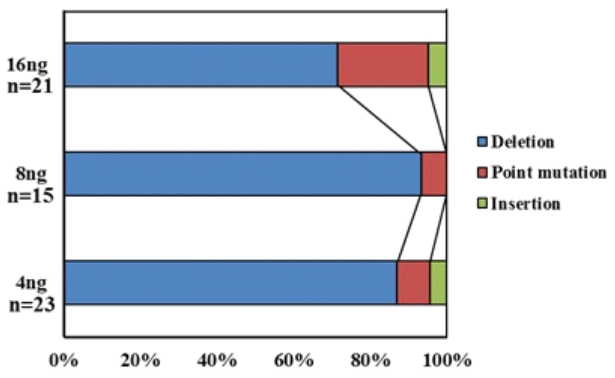
Table 2. Knockout efficiency of CRISPR/Cas9-mediated vector injected pig parthenotes.

Vector concentrations	No. of blastocysts examined	No. of colonies picked up and analyzed	No. (%)* of colonies mutated
4 ng/μl	11	125	23 (18.4 ± 4.2)
8 ng/μl	11	120	15 (12.5 ± 3.7)
16 ng/μl	11	105	21 (20.0 ± 2.5)

* Percentages are expressed as mean ± SEM of seven replicates.



(A)



(B)

Fig. 2. Representative mutation patterns (A) and rates (B) in FoxN1 loci of pig parthenotes derived by CRISPR/Cas9-mediated vector injection. The red hyphens, red letter with underline, and red letter denote deleted, point mutated, and inserted nucleotides, respectively.

genic animal is very useful for the study of medical and agricultural science (Whyte and Prather, 2011). Although, there are not enough researches on production of transgenic pigs in comparison to experimental animals such as mouse, rat, and so on. However, despite of present situation, some attempts to produce transgenic pigs were reported using several kinds of vector systems. For example, using ZFNs; α1,3-galactosyltransferase gene knockout pig (Hauschild *et al.*, 2011), PPARγ gene knockout pig (Yang *et al.*, 2011), and N-glycolylneuraminic acid gene knockout pig (Lutz *et al.*, 2013) and using TALENs; LDL receptor gene knockout pig (Carlson *et al.*, 2012), and finally vWF gene knockout pig using CRISPR/Cas9 system were reported (Hai *et al.*, 2014).

Recently developed meganucleases of ZFNs and TALENs are expensive to utilize, difficult to design, and required laborious cloning steps. The CRISPR/Cas9 system is reasonable to utilize because of its wide applications. Therefore, this system is receiving attention and becoming the next generation of engineered nucleases soon. The CRISPR/Cas9 system using the mouse (Wang *et al.*, 2013), rat (Li *et al.*, 2013), monkey (Niu *et al.*, 2014), human (Maeder *et al.*, 2013), drosophila (Yu *et al.*, 2013), zebra fish (Hwang *et al.*, 2013) and pig (Hai *et al.*, 2014) gene mutation has been verified successfully. Although, we used parthenogenetic embryos in this study, we could confirm the successful gene targeting by the combination of cytoplasmic microinjection skill and CRISPR/Cas9 system.

Using human codon-optimized *S. pyogenes* Cas9 with pX-330 vector, it has already been demonstrated previously in mouse study (Yang *et al.*, 2013). However, there is no report in case of pigs, and even in mammal. In the present study, normal operation of the humanized *S. pyogenes* Cas9 to modulate genome expression in pig parthenogenetic embryos is described for the first time.

To determine the optimal concentration of CRISPR/Cas9 vector for embryonic developmental competence, we could confirm that the concentration of CRISPR/Cas9 vector had no harmful effect on the embryonic development. In comparison between activation control group and injection penetration group, these two groups showed significant differences. Therefore, we concluded that the reduced developmental rate was originated from the procedure of cytoplasmic microinjection. In the previous study, it has already been reported about the cytoplasmic injuries occurred by the procedure of microinjection resulting decrease of developmental competence (Dumoulin *et al.*, 2001).

In xenotransplantation research field, usually, the gene-targeted somatic cells are used to generate multiple knockout cloned animals by SCNT technology. The CRISPR/Cas9 system showed one-step generation of transgenic mouse carrying multiple genes are reported recently (Yang *et al.*, 2013). However, the CRISPR/Cas9 system has several limitations. Firstly, the targetable spaces of SpCas9 are restricted by the necessity of a 5'-N20NGG-3' PAM sequence, which limits the target sites genome. Secondly, 6 mismatches between gRNAs and target DNA are tolerated by Cas9, which result in off-target effect (Jinek *et al.*, 2012). Additionally, a recent study demonstrated that the Cas9 nuclease induces mutations at off-target effect with 5 mismatches (Ding *et al.*, 2013).

In conclusion, we described that cytoplasmic microinjection of FoxN1-targeted CRISPR/Cas9 vector could efficiently generate transgenic pig parthenogenetic embryos in one-step. For the further study, we will try to produce transgenic pig by using *in vitro* fertilized embryo after CRISPR/Cas9 vector injection.

REFERENCES

Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, Voytas DF, Long CR, Whitelaw CB and Fahrenkrug SC. 2012. Efficient TALEN-mediated gene knockout in livestock. PNAS, USA. 109:17382-17387.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA and Zhang F. 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339:819-823.

Ding Q, Regan SN, Xia Y, Oostrom LA, Cowan CA and Musunuru K. 2013. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell 12:393-394.

Dumoulin JM, Coonen E, Bras M, Bergers-Janssen JM, Ignoul-Vanvuchelen RC, van Wissen LC, Geraedts JP and Evers JL. 2001. Embryo development and chromosomal anomalies after ICSI: effect of the injection procedure. Hum. Reprod. 16:306-312.

Gaj T, Gersbach CA and Barbas CF, 3rd. 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in Biotechnology 31:397-405.

Hai T, Teng F, Guo R, Li W and Zhou Q. 2014. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Research 24:372-375.

Hammer RE, Pursel VG, Rexroad CE, Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD and Brinster RL. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680-683.

Hauschild J, Petersen B, Santiago Y, Queisser AL, Carnwath JW, Lucas-Hahn A, Zhang L, Meng X, Gregory PD, Schwinzer R, Cost GJ and Niemann H. 2011. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. PNAS, USA. 108:12013-12017.

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR and Joung JK. 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature Biotechnology 31:227-229.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA and Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816-821.

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ and Prather RS. 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295:1089-1092.

Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, Li Y, Gao N, Wang L, Lu X, Zhao Y and Liu M. 2013. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nature Biotechnology 31:681-683.

Lutz AJ, Li P, Estrada JL, Sidner RA, Chihara RK, Downey SM, Burlak C, Wang ZY, Reyes LM, Ivary B, Yin F, Blankenship RL, Paris LL and Tector AJ. 2013. Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. Xenotransplantation 20:27-35.

Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH and Joung JK. 2013. CRISPR RNA-guided activation of endogenous human genes. Nature Methods 10:977-979.

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W and Sha J. 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156:836-843.

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F and Jaenisch R. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-me-

diated genome engineering. Cell 153:910-918.

Whyte JJ and Prather RS. 2011. Genetic modifications of pigs for medicine and agriculture. Mol. Reprod. Dev. 78:879-891.

Yang D, Yang H, Li W, Zhao B, Ouyang Z, Liu Z, Zhao Y, Fan N, Song J, Tian J, Li F, Zhang J, Chang L, Pei D, Chen YE and Lai L. 2011. Generation of PPARgamma mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. Cell Research 21:979-982.

Yu Z, Ren M, Wang Z, Zhang B, Rong YS, Jiao R and Gao G. 2013. Highly efficient genome modifications mediated by CRISPR/Cas9 in Drosophila. Genetics 195:289-291.

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