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Generation of Fibroblasts Lacking the Sal-like 1 Gene by Using Transcription Activator-like Effector Nuclease-mediated Homologous Recombination

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ABSTRACT: The Sal-like 1 gene (*Sall1*) is essential for kidney development, and mutations in this gene result in abnormalities in the kidneys. Mice lacking *Sall1* show agenesis or severe dysgenesis of the kidneys. In a recent study, blastocyst complementation was used to develop mice and pigs with exogenic organs. In the present study, transcription activator-like effector nuclease (TALEN)-mediated homologous recombination was used to produce *Sall1*-knockout porcine fibroblasts for developing knockout pigs. The vector targeting the *Sall1* locus included a 5.5-kb 5′ arm, 1.8-kb 3′ arm, and a neomycin resistance gene as a positive selection marker. The knockout vector and TALEN were introduced into porcine fibroblasts by electroporation. Antibiotic selection was performed over 11 days by using 300 μg/mL G418. DNA of cells from G418-resistant colonies was amplified using polymerase chain reaction (PCR) to confirm the presence of fragments corresponding to the 3′ and 5′ arms of *Sall1*. Further, mono- and bi-allelic knockout cells were isolated and analyzed using PCR–restriction fragment length polymorphism. The results of our study indicated that TALEN-mediated homologous recombination induced bi-allelic knockout of the endogenous gene. (**Key Words:** Knockout, Homologous Recombination, Transcription Activator-like Effector Nuclease [TALEN], Sal-like 1 Gene)

INTRODUCTION

Many patients with end-stage organ failure can only be treated using organ transplantation; however, transplantation is performed only in a limited number of patients because of a shortage of organ donors (Ye et al., 1994; Sprangers et al., 2008). Therefore, one of the ultimate goals of regenerative medicine is the generation of functional organs from stem cells (Liu et al., 2013). For stem cell replacement therapy, induced pluripotent stem cells (iPSCs) specific to patients can be generated using somatic cell nuclear transfer (SCNT) and reprogramming technology (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Recently, mice with rat pancreas were developed by injecting rat pluripotent stem cells (PSCs) into mouse blastocysts lacking the pancreatic and duodenal homeobox 1 gene (Kobayashi et al., 2010). The Sal-like 1 gene (*Sall1*) is essential for kidney development using studying *Sall1*-knockout mouse (Nishinakamura and Takasato, 2005). In a recent study, PSC-derived kidney was generated via blastocyst complementation, using *Sall1*-knockout blastocysts and mouse iPSCs or mouse embryonic stem cells (mESCs) (Usui et al., 2012). In another study, cloned pigs with exogenic pancreas were developed using blastocyst complementation (Matsunari et al., 2013). However, generation of PSC-derived kidneys in pigs has not been reported to date. The first requirement for producing exogenic kidneys in pigs is the development of heterozygous *Sall1*-knockout pigs.

Knockout pigs can be produced by performing SCNT in porcine fibroblasts (Dai et al., 2002). In general, knockout fibroblast can be produced using a gene-targeting system comprising knockout vectors. However, the efficiency for

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producing knockout fibroblasts is lower than that for producing knockout mESCs (Denning and Priddle, 2003; Laible and Alonso-González, 2009). Moreover, ESCs from domestic animals are not available easily. To overcome these problems, zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems have been used to develop knockout domestic animals (Carlson et al., 2012; Tan et al., 2013; Xin et al., 2013; Hai et al., 2014; Ni et al., 2014; Whitworth et al., 2014). Recently, although CRISPR/Cas9 system have been used to generate a knock-out pig, knock-out pigs were developed using TALEN with high efficient (Carlson et al., 2012; Xin et al., 2013; Yao et al., 2014). Targeting efficiency is very important for producing transgenic pigs via SCNT. Generation of knockout pigs via SCNT and transfected cells is associated with more advantages than generation of knockout pigs via DNA microinjection because the former method allows the assessment of vector integration in transfected cells before they can be used as nuclear donors (Wolf et al., 2000).

The aim of this study was to determine whether TALEN-mediated homologous recombination system on the *Sall1* gene locus in the porcine primary somatic cells is much more efficient than conventional homologous recombination system. We observed that use of TALEN-mediated homologous recombination resulted in the production of bi-allelic *Sall1*-knockout fibroblasts.

MATERIALS AND METHODS

Construction of knockout vector

Genomic DNA was isolated from the ear tissue of Chicago miniature pigs and the *Sall1* gene was amplified using polymerase chain reaction (PCR). The 5′ arm of the knockout vector was amplified using a forward primer with an additional NotI site (GCGGCCGCAATTGGCCTA CGAAAGAGGAGCAGCCTGTG) and a reverse primer with an additional BamHI site (GGATCCGATGG GGGCTGGCTCTTCGGTCTTGATGAA). The 3′ arm of the knockout vector was amplified using a forward primer with an additional SmaI site (CCCGGGATTCCAC AAGTCACCCAGCGCCGAGGAGAA) and a reverse primer with an additional SalI site (GTCGACGGA ATCTGTCTCAGCTCATATCCCAACACA). *PGK-neo*, which was used as a positive selection maker, was amplified from pKJ2-neo plasmid by using a forward primer with an additional BamHI site (GGATCCT ACCGGGTAGGGGAGGCGCTTTTC) and a reverse primer with an additional SmaI site (CCCGGGCCT CAGAAGAACTCGTCAAGAAGG). All the amplicons were subcloned into pGEM-T Easy vector (Promega Co., Madison, WI, USA), and their sequences were confirmed. The knockout vector was constructed as follows. The fragment corresponding to the 5′ arm (NotI–BamHI) was subcloned into pBluescript SK- (pBSK-) to produce a pBSK-5′-arm plasmid. The *PGK-neo* fragment (BamHI–SmaI) was then ligated into the BamHI–SmaI site of the pBSK-5′-arm plasmid to produce a pBSK-5′-arm-neo plasmid. The fragment corresponding to the 3′ arm was then inserted into the SmaI–SalI site of the pBSK-5′-arm-neo plasmid to produce a pBSK-5′-arm-neo-3′-arm plasmid. Finally, the pBSK-5′-arm-neo-3′-arm plasmid was digested using NotI–SalI and was ligated into the NotI–SalI site of pMCDT-A plasmid (Gibco BRL; Grand Island, NY, USA) to produce the *Sall1*-knockout vector. Thus, the knockout vector contained porcine *Sall1* 5′ arm (5.5 kb), *PGK-neo* as the positive selectable marker, porcine *Sall1* 5′ arm (1.8 kb), and a gene fragment encoding diphtheria toxin A (*DT-A*) as a negative selectable marker. For transfection into porcine fibroblasts, the *Sall1* knockout vector was digested with NotI.

TALEN and T7 endonuclease assay for validation

TALEN expression vector containing the CMV promoter that was designed to cleave the exon 2 of porcine *Sall1* was purchased from ToolGen, Inc. (Seoul, Korea). TALEN binding sites are shown in Figure 1A. For the validation of TALEN-mediated homologous recombination via a T7 endonuclease assay, porcine fibroblasts were transfected with the TALEN expression vector by electroporation and the genomic DNA of the transfected fibroblasts was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). The target region of TALEN was amplified from the genomic DNA by using gene-specific primers (sense: AGAAACCCAGGTGGGCTCCCGGAGGAGGCA; antisense: TGCATGCGGATGTGCTGCTGTAGGACCACA). The amplicons were purified using QIAquick Gel Extraction Kit (Qiagen; Hilden, Germany), denatured by heating, and annealed in hybridization buffer (10 mM Tris-HCl [pH 8.5], 75 mM KCl, and 1.5 mM $MgCl_2$) to produce heteroduplex DNAs. The heteroduplex DNAs were treated with T7 endonuclease I (4 U; New England Biolabs; Ipswich, MA, USA) at 37°C for 15 min. The resultant fragments were electrophoresed on a 2% agarose gel and were visualized by staining the gel with ethidium bromide (EtBr).

Culturing of fibroblasts and electroporation of the knockout vector

Porcine ear fibroblasts were prepared from tissues biopsied from the ear of specific pathogen-free Minnesota male miniature pigs maintained at the Seoul National University (Ahn et al., 2011). For electroporation, the fibroblasts were cultured in DMEM supplemented with

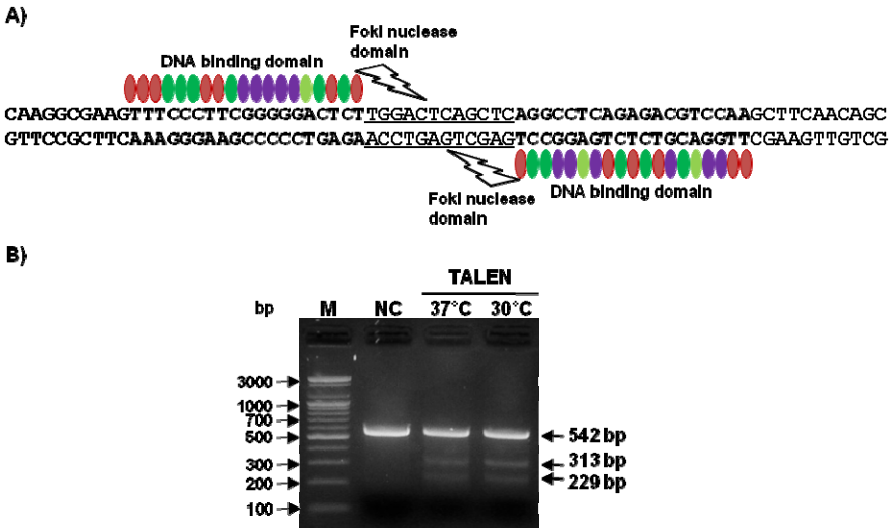

Figure 1. Binding site for TALEN and validation of TALEN activity. (A) The binding site of TALEN in exon 2 of porcine *Sall1*. (B) T7 endonuclease assay of porcine fibroblasts transfected with TALEN. TALEN, transcription activator-like effector nuclease; M, size markers; NC, negative control. Fibroblasts transfected with TALEN expression vector were incubated at 30°C and 37°C for 24 h.

15% defined fetal bovine serum (FBS), 1× non-essential amino acids, 1× sodium pyruvate, 10^{-4} M β-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Transfection was performed according to a previously reported method (Kim et al., 2012). Briefly, the fibroblasts were harvested by treatment with 0.25% trypsin and were resuspended in Ham's F10 medium (HyClone Co.; Logan, UT, USA) at a density of 5×10^6 cells per 0.4 mL of the medium. The fibroblast suspension (400 μL) was electroporated with 5 μg of the linearized knockout vector and each 2.5 μg of left and right TALEN expression vectors in a 4-mm cuvette by using four 1-ms pulses and 450-V capacitive discharges with BTX Electro-cell Manipulator (ECM 2001, BTX; Holliston, MA, USA). After electroporation, the cuvette was placed on ice for 10 min. The cells in the cuvette were resuspended in 10 mL of the medium, dispensed in a 48-well plate (800 cells/well), and cultured for 48 h. Cells harboring the knockout vector were selected over 10 to 12 days by culturing them in a medium containing 300 μg/mL geneticin-G418 (Gibco/Life Technologies, Gaithersburg, MD, USA). After selection, single colonies were passaged in 48-well plates and were cultured further for analysis.

Screening and PCR analysis of the knockout colonies

Knockout colonies were identified by performing the first and second PCRs. For the first PCR, 100 μL cell suspension from the 48-well plates was recovered by centrifugation. The cells were resuspended in 36 μL 50 mM NaOH. For genome extraction, the cells were incubated at 95°C for 10 min and were neutralized by treatment with 4 μL 1 M Tris-HCl (pH 8.0). The cells were then centrifuged at 10,000 rpm and 4°C for 10 min to obtain a crude genome extract. The first PCR was performed in a 50-μL reaction mixture containing 20 μL crude genome extract, 0.1 M each of *Neo* 3-2 sense (primer A: GCCTGCTTGCC GAATATCATGGTGGAAAAT) and *Sall1* Sc 5-2 antisense (primer B: GGGGAGAGGAAGGGAGAAGCTTAATAG TGG) primers for regions outside the 3′ arm, and KOD SYBR qPCR Mix (TOYOBO Co. Ltd., Osaka, Japan). Amplification was performed for 32 cycles of denaturation at 98°C for 30 s, annealing at 62°C for 30 s, and extension at 68°C for 2.5 min by using the Mx3000P qPCR System (Agilent Technologies, La Jolla, CA, USA).

Positive colonies from the first PCR were subcultured in 10-cm dishes. The cells were frozen in media containing 10% demethyl sulfoxide (DMSO) for further analyses. Genomic DNA of positive clones identified in the first PCR was isolated using GenElute Mammalian Genomic DNA Miniprep Kit and was used as the template for the second PCR to accurately identify the *Sall1*-knockout cells. The second PCR amplication was conducted using the 3′ arm, 5′ arms, and internal PCR. The 3′ arm was amplified using 100 ng genomic DNA under the same conditions as the first PCR. The 5′ arm was amplified using 100 ng genomic DNA, *Sall1* Sc Inl S primer (primer C: AAGCTGATTCAGATGCAGGCTTTTCCC) and *Neo* 5′-2 antisense primer (primer D: TGCTAAAGCGCATGCT CCAGACTGCCTTGG), and i-MAX II DNA Polymerase (iNtRON Biotechnology, Seongnam, Korea). Amplification was performed for 32 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s, and extension at 72°C for 6 min. To identify colonies with bi- and mono-allelic mutations, an internal PCR was performed using 100 ng genomic DNA, primers (primer E: GCCACCC

TCCAGTGGCAAAAGCGAAGACAG; primer F: TCATCGAAGGAGCCCGTGTCGGACTCCATG) against a part of exon 3 that was excluded from the knockout vector, and KOD FX Neo Polymerase (TOYOBO Co. Ltd., Japan). Amplification was conducted using a 2-step PCR involving 29 cycles of denaturation at 94°C for 10 s and annealing/extension at 68°C for 1 min. PCR products (20 μL) were validated by electrophoresis on a 0.8% agarose gel.

Validation of knockout colonies via PCR–RFLP

To validate the colonies showing mono- and bi-allelic mutations, PCR–restriction fragment length polymorphism (PCR–RFLP) was performed against a part of exon 3 that was excluded from the knockout vector. The PCR mixture included 100 ng genomic DNA, *Sall1* E2 Sc sense primer (primer H: AATCGTAAATGAGAGTCCAGCCTCTCCCCC) and *Sall1* Sc 5-2 antisense primer (primer B: GGGAGAGGAAGGGAGAAGCTTAATAGTGG), and KOD FX Neo Polymerase. Amplification was performed using a 2-step PCR involving 30 cycles of denaturation at 94°C for 30 s and annealing/extension at 68°C for 6 min. The PCR products were purified using QIAquick Gel Extraction Kit and were digested using SmaI. The digested PCR products (20 μL) were electrophoresed on 1% agarose gel to confirm the presence of targeted (2.8 and 1.8 kb) and non-targeted alleles (2.6 and 1.5 kb).

RESULTS

Validation of TALENs activity

In this study, we used TALEN to produce knockout fibroblasts. The target sequences of TALEN are located in exon 2 of *Sall1* (Figure 1A). To examine whether TALEN could be used for the targeted cleavage of endogenous *Sall1* in porcine fibroblasts, genomic DNA isolated from fibroblasts transfected with the TALEN expression vector was treated with T7 endonuclease I, which specifically cleaves heteroduplexes formed by the hybridization of wild-type and mutant DNA sequences. We observed that the T7 endonuclease I cleaved a fragment with an indel mutation in the genomic DNA isolated from fibroblasts transfected with the TALEN expression vector compared with genomic DNA isolated from negative controls, which were not transfected with the TALEN expression vector (Figure 1B). We examined the activity of TALEN at 30°C and 37°C because previous studies reported that the activity of TALENs is higher at 30°C than at 37°C. However, we observed no difference in the activity of the TALENs at these 2 temperatures.

Construction of the *Sall1* knockout vector

Figure 2 shows a schematic diagram of *Sall1* knockout strategy in porcine fibroblasts transfected with the *Sall1* knockout vector. The knockout vector contained a 5′ homologous arm (5.5 kb), *PGK-neo* as the positive selection marker, 3′ homologous arm (1.8 kb), and *DT-A* as the negative selection marker. The selection markers *PGK-neo* and *DT-A* did not contain a polyA signal and were only expressed if they were inserted at a gene locus before the polyA signal. This strategy allowed easy selection of knockout cells because cells with random integration of the knockout vector did not express *PGK-neo* and *DT-A* because of the absence of the polyA signal.

Targeting of the *Sall1* locus by using the knockout vector and TALEN-mediated homologous recombination

Efficiency of *Sall1* knockout by using TALEN-mediated homologous recombination is presented in Table 1. In all, 110 and 126 G418-resistant colonies transfected with and without the TALEN expression vector, respectively, and with the *Sall1* knockout vector were analyzed using the first PCR. Of these, 6 and 71 colonies, respectively, yielded positive results. The first PCR showed that TALEN-mediated homologous recombination along with the *Sall1* knockout vector had higher targeting efficiency (55.5%)

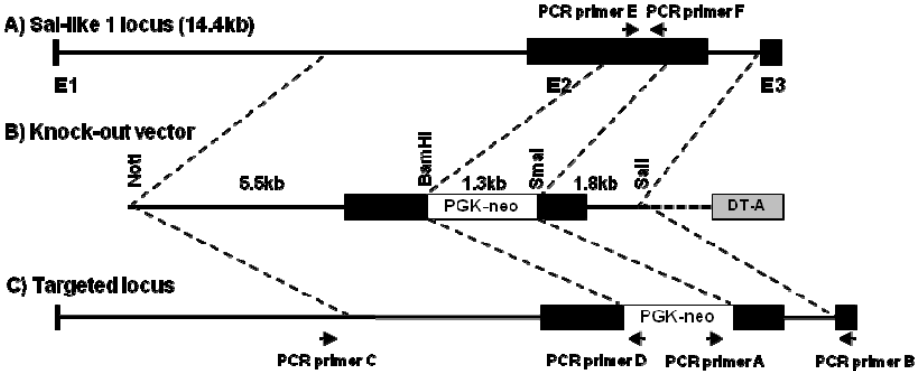

Figure 2. Strategy for the knockout of the porcine *Sall1* locus. (A) The *Sall1* locus, (B) the knockout vector, (C) the targeted locus. Solid black boxes indicate the exon. Arrows indicate polymerase chain reaction (PCR) primer pairs used for detecting homologous recombination.

Table 1. Gene targeting efficiency at the *Sall1* locus by TALEN-mediated homologous recombination using a knockout vector

TALEN	No. of transfected cells	No. of G418-resistant colonies analyzed by PCR	No. of positive colonies according to the first PCR	No. of colonies analyzed by 3′, 5′, and internal PCR	Wild–type colonies	Mono-allelic knockout colonies	Bi-allelic knockout colonies
–	5×10^6	110	6	4	4		
+	5×10^6	126	71	9	2	6	1
Total	1×10^7	236	77	13	6	4	1

TALEN, transcription activator-like effector nuclease; PCR, polymerase chain reaction.

than the knockout vector alone. In order to accurate analysis of homologous recombination, we analyzed 4 colonies in the without TALEN and 9 colonies in with TALEN by the 3′, 5′ and internal PCR analysis. Four colonies transfected with the *Sall1* knockout vector but not with the TALEN expression vector were all wild-type colonies. Of the 9 colonies transfected with both the *Sall1* knockout vector and the TALEN expression vector, 6 colonies (colony no. 22, 24, 27, 30, 46, and 58) were found to be mono-allelic knockout colonies and 2 colonies (colony No. 19 and 83) were found to be wild-type colonies. In addition, Colony number 5 in the Figure 3 in 9 colonies showed bi-allelic mutation (Table 1 and Figure 3). PCR–RFLP was conducted to validate the colonies showing bi- and mono-allelic mutations (Figure 4). Results of PCR–RFLP confirmed that only colony number 5 had bi-allelic mutations.

DISCUSSION

Recently, exogenic pancreas was developed in mice and pigs by performing blastocyst complementation with PSCs (Kobayashi et al., 2010; Matsunari et al., 2013). In another study, exogenic kidneys were developed in mice via blastocyst complementation, using injection of mouse iPSCs or mESCs into the *Sall1*-knockout blastocysts (Usui et al., 2012). These results raise the possibility that exogenic human kidney may be develop via blastocyst complementation using injection of human ES cell or iPSCs into the *Sall1*-knockout porcine blastocysts. *Sall1* is involved in kidney development (Nishinakamura and Takasato, 2005). To develop exogenic organs in pigs, it is important to develop heterozygous knockout pigs lacking specific genes required for kidney organogenesis.

In the present study, we produced *Sall1*-knockout porcine fibroblasts by using TALEN-mediated homologous recombination and *Sall1* knockout vector to using donor cells for producing *Sall1*-knockout pigs. The targeting efficiency (55.5%) was higher in the TALEN-mediated homologous recombination than in the conventional homologous recombination using knockout vector alone.

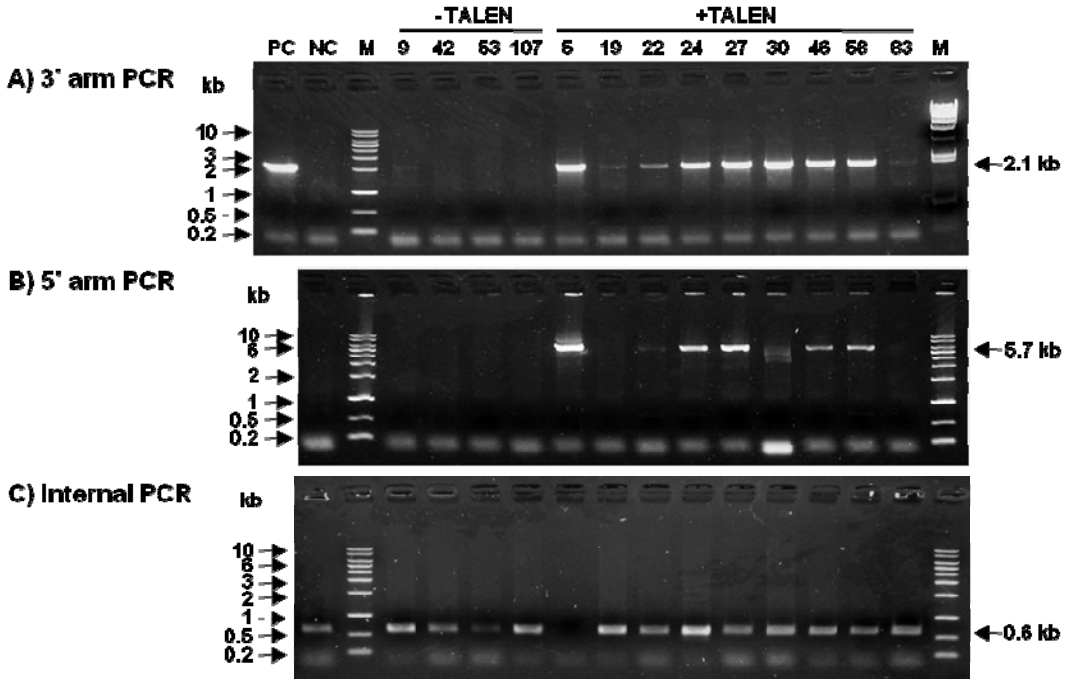

Figure 3. Analysis of *Sall1*-knockout fibroblasts via PCR. Representative EtBr-stained agarose gel showing PCR amplicons of (A) the 3′ arm, (B) the 5′ arm, and (C) internal PCR. PCR, polymerase chain reaction; EtBr, ethidium bromide; M, size marker (1-kb ladder); NC, negative control; PC, positive control; number, G418-resistant colonies.

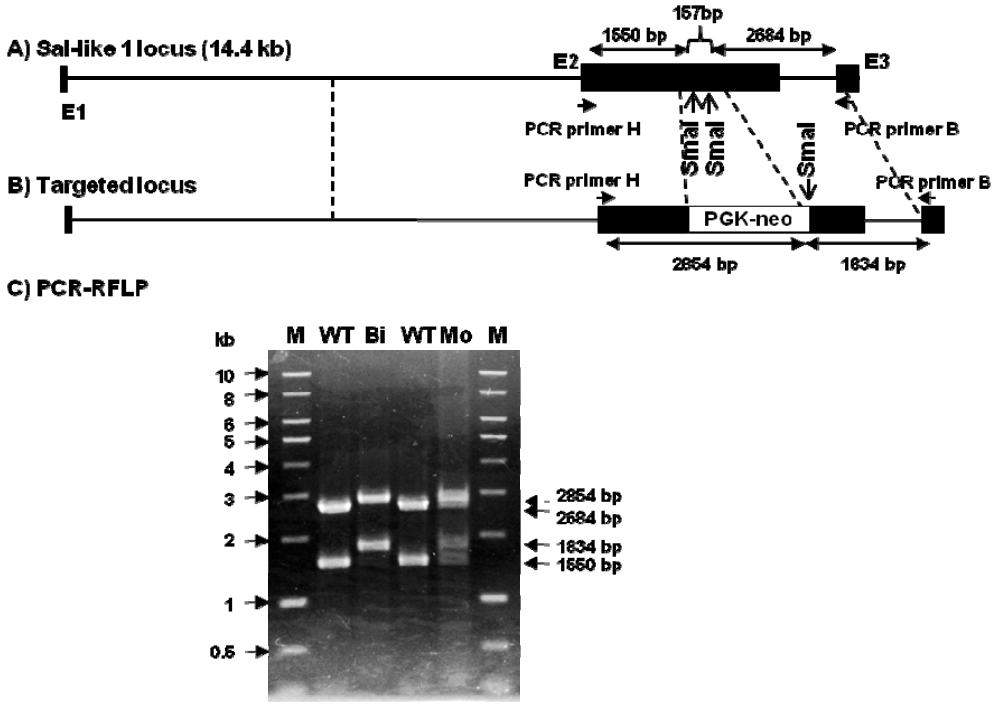

Figure 4. Analysis of colonies showing bi- and mono-allelic mutations by performing PCR–RFLP. (A) Diagram indicating the restriction enzyme site in the *Sall1* locus and (B) the targeted locus. (C) Representative EtBr-stained agarose gel showing amplicons obtained by PCR–RFLP. PCR–RFLP, polymerase chain reaction–restriction fragment length polymorphism; EtBr, ethidium bromide; M, size marker (1-kb ladder); WT, wild type; Mo, mono-allelic mutation; Bi, bi-allelic mutation.

Further, we confirmed that of the 7 *Sall1*-knockout fibroblast colonies, 6 and 1 colonies showed mono-allelic and bi-allelic mutations, respectively. Targeting efficiency of a conventional knockout vector for targeting the porcine α-1,3-galactosyltransferase locus was 0% to 9.3% in the absence of TALENs (Dai et al., 2002; Lai et al., 2002; Klumiuk et al., 2010). Moreover, the knockout efficiency of TALENs alone is higher than that of conventional methods (Carlson et al., 2012; Xin et al., 2013; Yao et al., 2014). Carson et al. (2012) reported 64% knockout efficiency by using TALENs alone in primary cells. Further, Xin et al. (2013) reported 89.5% knockout efficiency by using TALENs alone in porcine fetal fibroblasts. However, Yao et al. (2014) reported only 5% to 22% knockout efficiency by using TALENs alone in porcine fibroblasts. TALENs induce double-stranded DNA breaks in a specific target sequence, produce frameshift mutations, and silence the expression of target genes. The above authors used only TALENs against target genes to produce knockout cells. In the present study, we used TALEN-mediated homologous recombination along with a knockout vector to produce knockout fibroblasts. We observed low targeting efficiency because of the use of the knockout vector as a DNA donor for TALEN-mediated homologous recombination.

However, these results indicated that TALEN-mediated homologous recombination occurred more efficiently on the *Sall1* gene locus in the porcine primary somatic cells compare to that of conventional homologous recombination system. Also, fibroblasts with mono- and bi-allelic mutations obtained in this study could be used as nuclear donors for SCNT to develop *Sall1*-knockout pigs for producing exogenic kidneys by using PSCs.

CONFLICT OF INTEREST

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

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REFERENCES

Ahn, K. S., Y. J. Kim, M. Kim, B. H. Lee, S. Y. Heo, M. J. Kang, Y. K. Kang, J. W. Lee, K. K. Lee, J. H. Kim, W. G. Nho, S. S. Hwang, J. S. Woo, J. K. Park, S. B. Park, and H. Shim. 2011. Resurrection of an alpha-1,3-galactosyltransferase gene-targeted miniature pig by recloning using postmortem ear skin fibroblast. Theriogenology 75:933-939.

Carlson, D. F., W. Tan, S. G. Lillico, D. Stverakova, C. Proudfoot, M. Christian, D. F. Voytas, C. R. Long, C. B. Whitelaw, and S.

C. Fahrenkrug. 2012. Efficient TALEN-mediated gene knockout in livestock. Proc. Natl. Acad. Sci. USA. 109:17382-17387.

Dai, Y., T. D. Vaught, J. Boone, S. H. Chen, C. J. Phelps, S. Ball, J. A. Monahan, P. M. Jobst, K. J. McCreath, A. E. Lamborn, J. L. Cowell-Lucero, K. D. Wells, A. Colman, I. A. Polejaeva, and D. L. Ayares 2002. Targeted disruption of the alpha 1,3-galactosyltransferase gene in cloned pigs. Nat. Biotechnol. 20: 251-255.

Denning, C. and H. Priddle. 2003. New frontiers in gene targeting and cloning: success, application and challenges in domestic animals and human embryonic stem cells. Reproduction 126: 1-11.

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

Kim, J. W., H. M. Kim, S. M. Lee, and M. J. Kang. 2012. Porcine knock-in fibroblasts expressing hDAF on α-1,3-galactosyltransferase (GGTA1) gene locus. Asian Australas. J. Anim. Sci. 25:1473-1480.

Klumiuk, N., B. Aigner, G. Brem, and E. Wolf. 2010. Genetic modification of pigs as organ donors for xenotransplantation. Mol. Reprod. Dev. 77:209-221.

Kobayashi, T., T. Yamaguchi, S. Hamanaka, M. Kato-Itoh, Y. Yamazaki, M. Ibata, H. Sato, Y. S. Lee, J. Usui, A. S. Knisely, M. Hirabayashi, and H. Nakauchi. 2010. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. Cell 142:787-799.

Laible, G. and L. Alonso-González. 2009. Gene targeting from laboratory to livestock: Current status and emerging concepts. Biotechnol. J. 4:1278-1292.

Lai, L., D. Kolber-Simonds, K. W. Park, H. T. Cheong, J. L. Greenstein, G. S. Im, M. Samuel, A. Bonk, A. Rieke, B. N. Day, C. N. Murphy, D. B. Carter, R. J. Hawley, and R. S. Prather. 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295:1089-1092.

Liu, Y., R. Yang, Z. He, and W. Q. Gao. 2013. Generation of functional organs from stem cells. Cell Regen. 2:1

Matsunari, H., H. Nagashima, M. Watanabe, K. Umeyama, K. Nakano, M. Nagaya, T. Kobayashi, T. Yamaguchi, R. Sumazaki, L. A. Herzenberg, and H. Nakauchi. 2013. Blastocyst complementation generates exogenic pancreas *in vivo* in apancreatic cloned pigs. Proc. Natl. Acad. Sci. USA. 110:4557-4562.

Ni, W., J. Qiao, S. Hu, X. Zhao, M. Regouski, M. Yang, I.A. Polejaeva, and C. Chen. 2014. Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS One 9:e106718.

Nishinakamura, R. and M. Takasato. 2005. Essential roles of Sall1 in kidney development. Kidney Int. 68:1948-1950.

Sprangers, B., M. Waer, and A. D. Billiau. 2008. Xenotransplantation: Where are we in 2008? Kidney Int. 74: 14-21.

Takahashi, K. and S. Yamanaka. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka. 2007. Induction of pluriotent stem cells from adult human fibroblasts by defined factors. Cell 131:861-872.

Tan, W., D. F. Carlson, C. A. Lancto, J. R. Garbe, D. A. Webster, P. B. Hackett, and S. C. Fahrenkrug 2013. Efficient nonmeiotic allele introgression in livestock using custom endonucleases. Proc. Natl. Acad. Sci. USA. 110:16526-16531.

Usui, J., T. Kobayashi, T. Yamaguchi, A. S. Knisely, R. Nishinakamura, and H. Nakauchi. 2012. Generation of kidney from pluripotent stem cells via blastocyst complementation. Am. J. Pathol. 180:2417-2426.

Ye, Y., M. Niekrasz, S. Kosanke, R. Welsh, H. E. Jordan, J. C. Fox, W. C. Edwards, C. Maxwell, and D. K. Cooper. 1994. The pig as a potential organ donor for man. A study of potentially transferable disease from donor pig to recipient man. Transplantation 57:694-703.

Whitworth, K. M., K. Lee, J. A. Benne, B. P. Beaton, L. D. Spate, S. L. Murphy, M. S. Samuel, J. Mao, C. O'Gorman, E. M. Walters, C. N. Murphy, J. Driver, A. Mileham, D. McLaren, K. D. Wells, and R. S. Prather. 2014. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from *in vitro*-derived oocytes and embryos. Biol. Reprod. 91:78.

Wolf, E., W. Schernthaner, V. Zakhartchenko, K. Prelle, M. Stojkovic, and G. Brem. 2000. Transgenic technology in farm animals - progress and perspectives. Exp. Physiol. 85:615-625.

Xin, J., H. Yang, N. Fan, B. Zhao, Z. Ouyang, Z. Liu, Y. Zhao, X. Li, J. Song, Y. Yang, Q. Zou, Q. Yan, Y. Zeng, and L. Lai. 2013. Highly efficient generation of GGTA1 biallelic knockout inbred mini-pigs with TALENs. PLoS One 8:e84250.

Yao, J., J. Huang, T. Hai, X. Wang, G. Qin, H. Zhang, R. Wu, C. Cao, J. J. Xi, Z. Yuan, and J. Zhao. 2014. Efficient bi-allelic gene knockout and site-specific knock-in mediated by TALENs in pigs. Sci. Rep. 4 Article number: 6926.