Production of Cloned Pigs Derived from Double Gene Knockout Cells Using CRISPR/Cas9 System and MACS-based Enrichment System

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Abstract

Pigs are considered as optimal donor animal for the successful xenotransplantation. To increase the possibility of clinical application, genetic modification to increase compatibility with human is an important and essential process. Genetic modification technique has been developed and improved to produce genetically modified pigs rapidly. CRISPR/Cas9 system is widely used in various fields including the production of transgenic animals and also can be enable multiple gene modifications. In this study, we developed new gene targeting vector and enrichment system for the rapid and efficient selection of genetically modified cells. We conducted co-transfection with two targeting vectors for simultaneous inactivation of two genes and enrichment of the genetically modified cells using MACS. After this efficient enrichment, genotypic analysis of each colony showed that colonies which have genetic modifications on both genes were confirmed with high efficiency. Somatic cell nuclear transfer was conducted with established donor cells and genetically modified pigs were successfully produced. Genotypic and phenotypic analysis of generated pigs showed identical genotypes with donor cells and no surface expression of α-Gal and HD antigens. Furthermore, functional analysis using pooled human serum revealed dramatically reduction of human natural antibody (IgG and IgM) binding level and natural antibody-mediated cytotoxicity. In conclusion, the constructed vector and enrichment system using MACS used in this study is efficient and useful to generate genetically modified donor cells with multiple genetic alterations and lead to an efficient production of genetically modified pigs.

Key Words: Knockout Pig, Xenoantigens, Somatic Cell Nuclear Transfer, CRISPR/Cas9 system, MACS Based Enrichment

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**INTRODUCTION**

Genetic modification of pig to increase compatibility with human is an important process to increase the opportunity of clinical application of xenotransplantation. Targeted genetic modification in mice was reported more than 20 years ago firstly (Capecchi 2005). In contrast to mouse, generation of genetically modified pigs was difficult because of the lack of embryonic stem cells in the pigs. The reliance upon homologous recombination to generate targeted gene deletion and the need for breeding program to generate homozygous knockout pigs was major causes for the difficulty to generate genetically modified pigs. In 2002, α-Gal hetero knockout pigs were produced by the combination of genetically modified cells using homologous recombination and somatic cell nuclear transfer (SCNT) (Dai et al., 2002; Lai et al., 2002) and α-Gal homo knockout pigs were produced in 2003 using serial targeting and SCNT (Phelps et al., 2003). However, it was time- and cost-consuming process.

Genome editing technology can induce genomic modifications in specific gene locus using zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or clustered regularly interspaced palindromic repeats/CRISPR associated 9 (CRISPR/Cas9). Generation of genetically modified pigs has been advanced rapidly with genome editing system. In 2011 and 2013, generation of bi-allelic knockout pig of one or two xenoantigens using the ZFN and SCNT was reported (Hauschild et al., 2011; Lutz et al., 2013). Genome editing using CRISPR/Cas9 system is emerged in several years ago and widely used in various research fields (Hsu et al., 2014). It showed high efficiency, time- and cost-saving to generate genetically modified donor cells compared traditional technique such as homologous recombination. In addition, genetically modified pigs with multiple genetic modifications were successfully generated on relatively short periods (Butler et al., 2015), therefore, time and cost to generate genetically modified large animals have been dramatically reduced (Whitelaw et al., 2016).

In 2015, production of triple gene knockout pigs by SCNT with multiple genetic modified donor cells which is established by CRISPR/Cas9 targeting system and enrichment system using FACS sorting with carbohydrate binding lectin was reported (Estrada et al., 2015; Li et al., 2015). Although it is efficient strategy to establish the multiple genetic modified cells, FACS sorting with carbohydrate binding lectin to enrich the genetic modified cells has the limitation in universal application.

In this study, we modified Cas9 expressing vector with truncated H2Kk nucleotide sequences to enrich the genetic modified cells using MACS sorting system with H2Kk microbeads. Constructed vector was introduced into porcine primary fibroblasts to targeting GGTA1 and CMAH genes simultaneously. After enrichment using MACS sorting with H2Kk microbeads, multiple genetic modified cell colonies were harvested with high efficiency. In addition, GGTA1 and CMAH double knockout pigs were successfully generated by SCNT using established genetic modified cells. The results indicated that this developed vector and MACS sorting process in combination with the SCNT technique may be efficient and rapid for the generation of genetically engineered pigs.

**MATERIALS AND METHODS**

1. **Animals and animal care**

All pigs used in this study were housed at facilities in the biotechnology research institute of Mgenplus Co., Ltd. All animal experiments were approved by the Institutional Animal Care and Use Committee of the biotechnology research institute of Mgenplus Co., Ltd., Korea. All procedures were performed under the Committee guidelines. All surgical procedures were performed under general anesthesia and the necessary efforts were made to minimize any potential suffering of the animals. All pigs were raised under the conventional housing conditions.

2. **Design of sgRNA and Construction of targeting vector containing Cas9 and truncated mouse H2Kk**

To efficient enrichment, truncated mouse H2Kk coding nucleotide sequences for MACS sorting were linked with Cas9 CDS using T2A self-cleavage peptide allowing the each protein (Cas9 and truncated mouse H2Kk) to be translated simultaneously (bi-cistronic vector). Constructed bi-cistronic targeting vector was named by pCas9-H2Kk. Porcine GGTA1 and CMAH gene targeting single guide RNA (sgRNA) was designed by online CRISPR design tool (http://crispr.mit.edu/) and synthesized (Bioneer, Daejeon, Republic of Korea). Each sgRNA sequences as follows; 5’ - GAGAAAATAATGAATGTCAAAGG - 3’ for the GGTA1 exon 4 and 5’ - GAGAAAATAATGAATGTCAAAGG - 3’ for CMAH exon 3, respectively. Bold letter indicate PAM sequences in each sgRNA sequences. The synthesized oligomers were annealed and inserted into pCas9-H2Kk vector using BbsI restriction enzyme (NEB, MA, USA).
3. Establishment of *GGTA1* and *CMAH* double knock-out cells for the SCNT donor cells

Each 2 μg of constructed *GGTA1* and *CMAH* targeting pCas-H2Kk vectors were co-transfected into the porcine primary fibroblasts using Nucleofector™ (LONZA, Basel, Switzerland). After 48 h of transfection, enrichment was conducted by MACS using H2Kk microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The sorted cells were seeded on 100 mm cell culture dish by limiting dilution and cultured to establish the single cell-derived colonies. The single cell-derived colonies were picked and serially sub-cultured at 48, 24, 6-well plate, and 100 mm dish culture dishes. The cells were cryopreserved in liquid nitrogen and used as donor cells to SCNT.

4. Confirmation of genetic mutation on the target site of *GGTA1* and *CMAH* gene

The genomic DNA of the each single cell-derived colony was extracted using commercial gDNA extraction kit (iNtRon Biotechnology, Seongnam, Republic of Korea) following the manufacturer’s protocol. To check genetic modifications on the target gene site, nested PCR was performed using *Pfu* Plus 5x master mix (ELPIS biotech, Daejeon, Republic of Korea). 1st PCR was conducted 30 cycles with 1st PCR primer sets of *GGTA1* (F: 5’ - GGGATGCTAGCAGTCTGGA - 3’, R: 5’ - GACGGTAATTTAAGGGCTCAGG - 3’) and *CMAH* (F: 5’ - GGAGAGGGAACGACAGAC - 3’, R: 5’ - GAAATGAGGCAACGAGAC - 3’) following condition; 5 min of denaturation at 95 °C, reacting 30 cycles (95 °C for 30s, 58 °C for 30s, 72 °C for 1 min), and last extension at 72 °C for 10 min. Then, 2nd PCR was carried out 30 cycles using 1st PCR products (1:100 dilution) as templates with 2nd PCR primer sets of *GGTA1* (F: 5’ - CCTCTAGTCTGTGATGCGAGG - 3’, R: 5’ - AGCTGGTGACTTGGCT-GATAA - 3’) and *CMAH* (F: 5’ - CCTGGGAGCTGTCAATGCTC - 3’, R: 5’ - AATAGTTCTTCAAGGAGAGAGGCAACGACAGAC - 3’)) following condition; 5 min of denaturation at 95 °C, reacting 30 cycles (95 °C for 30s, 58 °C for 30s, 72 °C for 1 min), and last extension at 72 °C for 10 min.

Then, 2nd PCR amplicons were used for T7E1 assay. Amplicons were denatured at 95°C for 5 min and re-annealed at -2°C/sec to 85°C, -0.1°C/sec to 25°C, then T7E1 (ToolGen, Seoul, Korea) enzyme treated into re-annealed products at 37°C for 30 min. The 2nd PCR products and T7E1 digested products were loaded on 2% agarose gel in TAE buffer and electrophoresed on 100v 25min. Then, using the UV trans-illuminator (BIO RAD Laboratories Inc, CA, USA), the electrophoresed gels were visualized. The nucleotide sequences of the 2nd PCR products were analyzed by Sanger sequencing method (Macrogen, Seoul, Korea).

5. Somatic cell nuclear transfer and Embryo transfer

The SCNT procedures were followed as described in previous studies (Kang et al., 2016a; Kang et al., 2016b; Kang et al., 2016c). Briefly, the oocytes were collected from local slaughterhouses and transported to laboratory at 25–30°C normal saline (N/S) containing 1% (v/v) Pen Strep (Thermo Fisher Scientific, Gibco, CA, USA). After 2–3 times washing with N/S, cumulus oocyte complexes (COC) were aspirated from antral follicles (3–6mm in diameter) using 18-gauge needle syringe. The COCs were washed in modified Tyrode’s solution (TALP)-Hepes and cultured in maturation media based tissue culture media 199 (TCM-199, Thermo Fisher Scientific) supplemented with 0.57 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/ml EGF, 1X Insulin-Transferrin-Selenium-A Supplement (ITS-A, Thermo Fisher Scientific), 1% (v/v) pen-strep, 0.5 μg/ml follicle stimulating hormone (FSH), 0.5 μg/ml luteinizing hormone (LH) and 10% porcine follicular fluid. After 22 h of culture, the cultured COCs were transferred to IVM medium without FSH and LH for additional 22 h. The COCs were cultured at 38 °C with 5% CO2. After maturation, COCs were denuded by pipetting with 0.1% hyaluronidase in TCM-Hepes. The matured oocytes were enucleated by finely manufactured glass pipette (16-18um in diameter) in manipulation medium with 5 μg/ml cytochalasin B. A single cell was injected into perivitelline space of enucleated oocyte by manipulator. Then cell-injected oocytes were fused and activated by electric pulse (BTX, two DC pulses of 1.2 kV / cm for 60 μsec). Activated embryos were incubated with 0.5μM Scriptaid, a histone deacetylase inhibitor, for overnight. Then, the reconstructed embryos were cultured in porcine zygote medium 3 (PZM3) in 5% CO2 at 39 °C until transferring to a surrogate. The surrogates were induced to estrus synchronization by Regumate-PG600 administration system before embryos transfer. The reconstructed embryos were surgically transferred into surrogate’s oviduct by Tamcat catheter (Covidien, Dublin, Ireland). Successful implantation was detected by ultrasound at 4 weeks after embryo transfer. After pregnancy period, cloned piglets were delivered by C-section or normal delivery from surrogate.
6. Characterization of genetic modified piglets

Total 12 piglets including 3 stillbirths were delivered by two surrogates. Four of nine live piglets were died in few days after birth because of unknown causes. Remained 5 piglets were used to confirm the genotypic, phenotypic, and functional analysis.

1) Genotypic analysis

The genomic DNA from each piglet was isolated and used for genotypic analysis. Nested PCR of target region of both genes and T7E1 assay was conducted following the method mentioned above.

2) Phenotypic analysis

The expression of α-Gal and HD antigens was confirmed by flow cytometry using PBMCs. Whole blood was collected from each piglet after 2 weeks of birth and PBMCs were isolated from the whole blood using Ficoll-Paque™ PLUS (GE Healthcare, IL, USA). Isolated PBMCs were stained with FITC-conjugated isolectin B4 (1:100, Enzo Life Science, NY, USA) for α-Gal or Anti-Neu5Gc antibody (1:200, BioLegend, CA, USA) and Alexa Fluor®647-conjugated AffiniPure F(ab’)2 Fragment Donkey anti-chicken IgY (1:100, Jackson ImmunoResearch, PA, USA) for HD antigens. Flow cytometry was conducted by BD Accuri™ C6 PLUS (BD Biosciences, CA, USA).

3) Functional analysis

Natural antibody binding level to α-Gal and HD antigens was analyzed by flow cytometry using heat-inactivated pooled human serum (Sigma Aldrich, MO, USA). Briefly, 40% heat-inactivated human serum was treated to primary fibroblasts of each piglet for 20 min. After three times wash, FITC-conjugated anti-human IgG (F9512) or FITC-conjugated anti-human IgM (F5384, Sigma Aldrich, MO, USA) was treated for 20 min at 4 °C in dark. Then, three times wash was carried out and flow cytometry was conducted.

Natural antibody-mediated cytotoxicity was also analyzed using pooled human serum (normal or heat-inactivated). Briefly, 40% human serum was treated to primary fibroblasts of each piglet for 30 min at 37 °C. After three times wash, 7-AAD (BD Pharmingen™, CA, USA) was treated for 5 min at room temperature in dark. Then, after three times wash again, flow cytometry analysis was also conducted.

RESULTS

1. Construction of targeting vector and establishment of GGTA1 and CMAH double knock-out cells

To efficient enrichment, targeting vector was modified using T2A self-cleavage peptide and extracellular domain of H2Kk for MACS sorting. Truncated H2Kk gene was inserted after the Cas9 CDS with T2A self-cleavage peptide using restriction enzyme. Constructed vector was named as pCas9-H2Kk (Figure 1A). sgRNA for the exon 4 of porcine GGTA1 and the exon 3 of porcine CMAH gene was synthesized and inserted into the pCas9-H2Kk vector, respectively (Figure 1B). A constructed targeting vector was co-transfected to porcine primary fibroblasts by electroporation and sorted by H2Kk microbeads after 48 h of transfection. Total 32 single cell-derived colonies were picked and 28 single cell-derived colonies were analyzed by PCR and T7E1 assay. Among the analyzed colonies, 12 colonies showed genetic modifications on both genes (Figure 1C). After enrichment using H2Kk microbeads, the targeting efficiency on the both genes was 42.9% (Table 1). Finally, in three colonies (#2, #29, and #30), Sanger sequencing analysis was conducted to confirm the exact indels. Colony #2 was shown 2 base pair (bp) deletion and 1 bp insertion on the GGTA1 target locus and 1 bp insertion (T or C) on the CMAH target locus, #29 was shown 25 bp deletion and 1 bp deletion on the GGTA1 target locus and 12 bp deletion and 1 bp insertion on the CMAH target locus, and #30 was shown 2 bp deletion and 1 bp insertion on the GGAT1 target locus and 3 bp deletion and 1 bp insertion on the CMAH target locus (Figure 1D).

Analyzed three colonies were shown bi-allelic hetero mutation on the both genes (GGTA1 and CMAH).

2. Production of GGTA1 and CMAH double knock-out piglets by SCNT

Established GGTA1/CMAH gene modified cells (colony #2) was used as donor cells for the SCNT. Total 773 re-constructed embryos were transferred into oviduct of 4 surrogates. After 28–30 days of embryo transfer, early implantation was detected by ultrasound. Three surrogates had pregnancy and two of them maintained to full-term period (≥ 114 days). Total 12 piglets were delivered including 3 stillbirths from two surrogates (Table 2, Figure 2A). Four of nine piglets were died in few days by unknown causes. Remained 5 piglets were used to genotypic or phenotypic characterization. Tail tissue and whole blood were collected from these piglets for the primary cell cultures and the characterization.
Figure 1. Construction of targeting vector and Establishment of GGTA1/CMAH double knock-out cells.
A) Schematic diagram of pCas9-H2Kk vector. B) Genomic target site of porcine GGTA1 and CMAH genes. Target site for GGTA1 gene is located in exon 4 and for CMAH gene is located in exon 3. sgRNA sequences is bolded and PAM sequences (NGG) indicated by underline. C) PCR of target site on both genes and T7E1 assay in each single cell-derived colony. Single cell-derived colonies which have genetic modifications on the both genes are represented by red character. D) Result of Sanger sequencing analysis. Selected colonies among the colonies which have genetic modifications on the both genes were conducted Sanger sequencing to confirm the precise indels. sgRNA target site is represented by bold and PAM sequences are indicated by underline. Deletion of nucleotides is represented by dashed line and insertion of nucleotide is represented by red character.

Table 1 Targeting efficiency after enrichment using MACS sorting with H2Kk microbeads

| No. of Colonies (picked) | No. of Colonies (analyzed) | No. of Colonies with mutations on the both gene (%) |
|--------------------------|----------------------------|-----------------------------------------------|
| 32                       | 28                         | 12 (42.9)                                     |

Table 2 Efficiency of GGTA1/CMAH double KO pig production by SCNT

| Recipient | No. of transferred embryo | Pregnancy | Delivery | No. of piglets | Cloning efficiency (%) |
|-----------|----------------------------|-----------|----------|----------------|------------------------|
| 1         | 255                        | Y         | Y        | 1              | 1.2                    |
| 2         | 211                        | Y         | N        | 0              | 0                      |
| 3         | 162                        | N         | -        | 0              | 0                      |
| 4         | 145                        | Y         | Y        | 2              | 6.2                    |
| Total     | 773                        | 3/4 (75%) | 2/3 (66.7%) | 3              | 1.6                    |

J. Emb. Trans. (2018) Vol. 33, No. 4
3. Characterization of generated piglets

To confirm the genotypic character of generated piglets, nested PCR of target region of both genes and T7E1 assay was conducted. The exact genetic indels were confirmed by Sanger sequencing analysis of target region of both genes. Furthermore, the surface expression of α-Gal and HD antigens was confirmed by flow cytometry using fresh isolated PBMCs. Finally, human natural antibody binding affinity and antibody-mediated cytotoxicity were evaluated to confirm the functional aspect of GGTA1/CMAH double knock-out pigs.

1) Genotypic analysis

To confirm the genetic modifications in the target region of the GGTA1 and CMAH gene, genomic DNA was isolated from each piglet and nested PCR and T7E1 assay was conducted. The genomic DNA isolated from two piglets (1-1 and 1-2) was shown identical genetic modification patterns with donor cells (Figure 2B) and other genomic DNA isolated from three piglets (4-1, 4-2, and 4-3) was also shown identical genetic modification patterns (data not shown). In addition, Sanger sequencing analysis of target region in the genomic DNA isolated from five piglets (1-1, 1-2, 4-1, 4-2, and 4-3) revealed 2 bp deletion and 1 bp insertion on the GGTA1 gene and 1 bp insertion (T or C) on the CMAH gene like donor cells (Figure 2C). As a result, all of analyzed piglets were confirmed as the bi-allelic hetero mutations in each gene (GGTA1 and CMAH).
2) Phenotypic analysis

It is well known that the HD antigens are existed in the serum originated from animal such as fetal bovine serum. Therefore, fresh isolated PBMCs were used to confirm the surface expression of α-Gal or HD antigens instead of primary cultured fibroblasts. PBMCs were isolated from whole blood of three generated piglets (4-1, 4-2, and 4-3). Flow cytometry was performed to confirm the expression of α-Gal or HD antigens on the surface of isolated PBMCs. All analyzed PBMCs from three piglets did not express α-Gal and HD antigens on the cell surface, suggesting the complete knockout of the GGTA1 and CMAH gene in these piglets (Figure 3).

3) Functional analysis

α-Gal and HD antigen is representative natural antigen to induce severe immune rejection as known as hyperacute rejection in xenotransplantation model. During the hyperacute rejection, natural antibody mediated immune responses happen mainly. Therefore, the evaluation of reduction of human natural antibody binding level and antibody-mediated cytotoxicity in GGTA1/CMAH double knock-out pig is important functional aspect. First, heat-inactivated pooled human serum (40%) was treated to primary cultured fibroblasts from generated piglets (1-1, 1-2, 4-1, 4-2, and 4-3) and the natural antibody (IgG and IgM) binding level was checked by flow cytometry. All analyzed cells showed dramatic reduction of antibody binding level (Figure 4A and B). Next, to confirm the antibody-mediated cytotoxicity, pooled human serum (40%) was also treated to primary cultured fibroblasts and the level of cytotoxicity was checked using 7-AAD and flow cytometry. As expected, cytotoxicity was decreased in all of GGTA1/CMAH double knock-out cells (Figure 4C).

Taken together, generated piglets have bi-allelic hetero mutation in target region of both genes (GGTA1 and CMAH) and showed no surface expression of α-Gal and HD antigens. Functionally, GGTA1 and CMAH double knock-out cells showed dramatic reduction of xeno-reactive natural antibody binding level and antibody-mediated cytotoxicity. Therefore, in this study, GGTA1/CMAH double knock-out piglets were successfully generated by SCNT using the donor cells established with efficient enrichment procedure.

Figure 3. Surface expression of xeno-antigens on the PBMCs isolated from GGTA1/CMAH double knockout piglets. Flow cytometry was conducted to confirm the expression of xeno-antigens using the PBMCs. Black histogram indicates non-treated control for α-Gal or isotype control for HD antigens.
The generation of genetically modified pigs is time- and cost-consuming process. The combination of CRISPR/Cas9 system and somatic cell nuclear transfer technique can improve the efficiency of transgenic pig production (Butler et al., 2015). The successful and effective generation of genetically modified pigs is dependent on the effective and precise development of genetically modified donor cells. CRISPR/Cas9 system is powerful tool to induce precise genetic modifications effectively in the development of genetically modified donor cells for SCNT. In order to increase the efficiency of genetic modifications in cells, antibiotics selection or cell sorting using fluorescence protein can be used (Ran et al., 2013). Although antibiotics selection is conventionally easy and useful methods to enrich the genetic modified cells, the treatment of antibiotics may be harmful to the cells. Also, antibiotics selection requires more times than cell sorting system to separate cells which have genetic modifications. Generally, more than 3 days of antibiotics treatment is required for efficient separation of genetic modified cells. Whereas cell sorting system require few hours to separate efficiently genetic modified cells. FACS sorting by laser and MACS sorting by magnetic microbeads are widely used to separate cells. Cells separated through FACS sorting might get potentially damaged as being exposed to various stress including hydraulic pressure, shear force, vibrations, and high voltages during flow cytometry (Wolff et al., 2003). In addition, FACS sorting must require expensive equipment such as cell sorter. In this respect, MACS based enrichment system can be relatively less harmful to the cells and easily applicable compared with antibiotics selection and FACS mediated enrichment.

In this study, we modified sgRNA and Cas9 expressing vector to increase the efficiency of genetic modified donor cell development using T2A self-cleavage peptide and truncated H2K4 nucleotide sequences for MACS sorting. To evaluate the efficiency of the development of donor cells, we tried to establish the GGTA1 and CMAH double knockout cells using constructed targeting vector and magnetic sorting system. After the enrichment using magnetic sorting system, total 28 single cell-derived colonies were analyzed genotypic character of both target genes. As a result, the ratio of colonies which have the mutations on the both genes was revealed high efficiency (12/28, 42.9%).

Among the colonies, colony #2 carrying the bi-allelic hetero
mutations on the GGTA1 and CMAH gene was used for the generation of the GGTA1 and CMAH double knockout pigs by SCNT and the healthy piglets were produced successfully. Genotypic analysis of generated piglets showed identical mutation with donor cells (#2) and phenotypic analysis showed no surface expression of α-Gal and HD antigens as expected. Furthermore, the binding level of human natural antibody (IgG and IgM) and human natural antibody-mediated cytotoxicity was reduced dramatically. Slightly higher antibody binding level compared with control might be the effect of residual natural antibody to react other xeno-antigens except α-Gal and HD antigens. However, there are no differences in antibody-mediated cytotoxicity between the heat-inactivated human serum treated control and the human serum treated group. It means that residual xeno-reactive natural antibodies are not major players in hyperacute immune responses.

In conclusion, in this study, we developed new enrichment method by MACS sorting system using H2Kk microbeads to increase the efficiency of the development of genetic modified donor cells which have genetic modifications of two target genes. In addition, GGTA1 and CMAH double knockout piglets were successfully generated by SCNT using the developed donor cells and characterization of generated piglets was confirmed by genotypic, phenotypic, and functional analysis. Gene targeting vector containing sRNA, Cas9 CDS, and truncated H2Kk nucleotide sequences which constructed in this study can be useful to establish genetic modified cells with single gene modifications as well as multiple gene modifications through relatively simple sorting system.

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CONFLICT OF INTEREST

BC, SJK, EJL, SMA, JSL, DYJ, SHL and JTK are employees of Mgenplus Co., Ltd., a company that specializes in transgenic pig production.

REFERENCES

Butler JR, Ladowski JM, Martens GR, Tector M and Tector AJ. 2015. Recent Advances in Genome Editing and Creation of Genetically Modified Pigs. Int. J. Surg. 23(Pt B):217 - 222.

Capecchi MR. 2005. Gene targeting in mice: Functional analysis of the mammalian genome for the twenty-first century. Nat. Rev. Genet. 6:507-512

Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA and Ayares DL. 2002. Targeted disruption of the α1, 3-galactosyltransferase gene in cloned pigs. Nat. Biotechnol. 20:251-255.

Estrada JL, Martens G, Li P, Adams A, Newell KA, Ford ML, Butler JR, Sidner R, Tector M and Tector AJ. 2015. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/β4GalNT2 genes. Xenotransplantation. 22:194-202.

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. Proc. Natl. Acad. Sci. 108:12013-12017.

Hsu PD, Lander ES and Zhang F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 157:1262 - 1278.

Kang JT, Cho B, Ryu J, Ray C, Lee EJ, Yun YJ, Ahn S, Lee J, Ji DY, Jue N, Clark-Deener S, Lee K and Park KW. 2016a. Biallelic modification of IL2RG leads to severe combined immunodeficiency in pigs. Reprod. Biol. Endocrinol. 14:74.

Kang JT, Ryu J, Cho B, Lee EJ, Yun YJ, Ahn S, Lee J, Ji DY, Lee K and Park KW. 2016b. Generation of RUNX3 knockout pigs using CRISPR/Cas9-mediated gene targeting. Reprod. Domest. Anim. 51:970-978.

Kang JT, Kwon DK, Park AR, Lee EJ, Yun YJ, Ji DY, Lee K and Park KW. 2016c. Production of α1,3-galactosyltransferase targeted pigs using transcription activator-like effector nucleasemediated genome editing technology. J. Vet. Sci. 17:89 - 96.

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 α-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science. 295:1089-1092.

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 α-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science. 295:1089-1092.

Li P, Estrada JL, Burlak C, Montgomery J, Butler JR, Santos RM, Wang ZY, Paris LL, Blankenship RL, Downey SM, Tector M
Production of double carbohydrate xenoantigens knock-out cloned pigs

and Tector AJ. 2015. Efficient generation of genetically distinct pigs in a single pregnancy using multiplexed single-guide RNA and carbohydrate selection. Xenotransplantation. 22:20-31.

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

Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y and Ayares DL. 2003. Production of α1,3-galactosyltransferase-deficient pigs. Science. 299:411-414.

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA and Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8:2281-2308.

Whitelaw CB, Sheets TP, Lillico SG and Telugu BP. 2016 Engineering large animal models of human disease. J. Pathol. 238:247-256.

Wolff A, Perch-Nielsen IR, Larsen UD, Friis P, Goranovic G, Poulsen CR, Kutter JP and Tellemann P. 2003. Integrating advanced functionality in a microfabricated high-throughput fluorescent-activated cell sorter. Lab Chip. 3:22-27.