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.

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INTRODUCTION

Genetic modification of pig to increase compatibility with human is 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 H2K^k nucleotide sequences to enrich the genetic modified cells

using MACS sorting system with H2K^k microbeads. Constructed vector was introduced into porcine primary fibroblasts to targeting *GGTA1* and *CMAH* genes simultaneously. After enrichment using MACS sorting with H2K^k 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 H2K^k

To efficient enrichment, truncated mouse H2K^k coding nucleotide sequences for MACS sorting were linked with Cas9 CDS using T2A self-cleavage peptide allowing the each protein (Cas9 and truncated mouse H2K^k) to be translated simultaneously (bi-cistronic vector). Constructed bi-cistronic targeting vector was named by pCas9-H2K^k. 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' - GAGAAATAATGAATGTCAAAGG - 3' for the *GGTA1* exon 4 and 5' - GAAGAATAAGAGCCGCCTGAAGG - 3' for *CMAH* exon 3, respectively. Bold letter indicate PAM sequences in each sgRNA sequences. The synthesized oligomers were annealed and inserted into pCas9-H2K^k 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-H2K^k 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) as 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' - GGCTAGTGGCTACAGCTCTGA - 3', R: 5' - GACGGTATTTAAGGGCTCAGG - 3') and *CMAH* (F: 5' - GGAGAGAGGCAACGACAGAC - 3', R: 5' - GAAATGATCAGAAGAGCCGC - 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 m), and last extension at 72 °C for 10 min. Then, 2nd PCR was carried out 30cycles using 1st PCR products (1:100 dilution) as templates with 2nd PCR primer sets of *GGTA1* (F: 5' - CCTCTAGTCTGTGATGCGAGG - 3', R: 5' - AGCTGGTGAAGTGGCT-GATAA - 3') and *CMAH* (F: 5' - CCTGTGGAGCTGTCAATGCTC - 3', R: 5' - AATAGTTCTTCA GAGAG-GATG - 3') as following condition; 5 min of denaturation at 95 °C, reacting 30 cycles (95 °C for 30s, 58 °C for 30s, 72 °C for 30s), and last extension at 72 °C for 10 min. The second 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 ovaries 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 2~3times 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 (16-18µm 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 0.5uM 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-PaqueTM 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 AccuriTM 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 PharmingenTM, 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 H2K^k for MACS sorting. Truncated H2K^k gene was inserted after the Cas9 CDS with T2A self-cleavage peptide using restriction enzyme. Constructed vector was named as pCas9-H2K^k (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-H2K^k vector, respectively (Figure 1B). A constructed targeting vector was co-transfected to porcine primary fibroblasts by electroporation and sorted by H2K^k 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 H2K^k 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 *GGTA1* 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 *GGT1*/*CMAH* double knock-out cells.

A) Schematic diagram of pCas9-H2K^k vector. B) Genomic target site of porcine *GGT1* and *CMAH* genes. Target site for *GGT1* 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 H2K^k 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 *GGT1*/*CMAH* double KO pig production by SCNT

Recipient	No. of transferred embryo	Pregnancy	Delivery	No. of piglets		Cloning efficiency (%)
				Stillbirth	Live	
1	255	Y	Y	1	2	1.2
2	211	Y	N	0	0	0
3	162	N	-	0	0	0
4	145	Y	Y	2	7	6.2
Total	773	3/4 (75%)	2/3 (66.7%)	3	9	1.6

Figure 2. Production of genetically modified piglets and genotypic analysis. A) Representative picture of produced *GGTA1/CMAH* double knockout piglets. B) PCR and T7E1 assay of target site on the both genes. Arrowhead indicates cleaved DNA by T7E1 enzyme. C) Sanger sequencing results of target site on the both genes. Bold character indicates sgRNA target region and underline indicates PAM sequences. Red line represents double-strand break region by Cas9 protein and red box represent PAM sequences. Dashed line represents deletion of nucleotides and red character represent insertion of nucleotides.

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 piglets 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 4 A 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.

Figure 4. Protective effects of *GGTA1/CMAH* double knockout cells against human natural antibody binding and antibody-mediated cytotoxicity. A) and B) The level of human natural antibody binding (IgG and IgM) is evaluated by flow cytometry. Heat-inactivated human serum was treated to porcine primary fibroblasts isolated from each piglet including age-matched wildtype piglet. Heat-inactivated human serum dose not treated to the control group named 2nd Only, meaning only secondary antibody binding. C) Human natural antibody-mediated cytotoxicity is also evaluated by flow cytometry. Heat-inactivated human serum is used as negative control.

DISCUSSION

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 H2K^k 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 H2K^k 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 H2K^k 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, EKL, SMA, JSL, DYJ, SHL and JTK are employees of Mgenplus Co., Ltd., a company that specializes in transgenic pig production.

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