·医药生物技术·

敲除 GGTA1 同时表达人白细胞抗原 G5 的基因修饰猪的构建

周小青[1,2#], 刘玉[1#], 唐成程[1,2], 程令印[1], 郑淑文[1], 郑雨龄[1], 陈敏[1,2], 杨化强[3], 邹庆剑[1,2], 赖良学[1,4]

1 五邑大学 生物科技与大健康学院, 广东 江门 529020
2 广东省医学大动物模型重点实验室, 广东 江门 529020
3 华南农业大学 动物科学学院, 广东 广州 510642
4 中国科学院广州生物医药与健康研究院, 广东 广州 510530

周小青, 刘玉, 唐成程, 程令印, 郑淑文, 郑雨龄, 陈敏, 杨化强, 邹庆剑, 赖良学. 敲除 GGTA1 同时表达人白细胞抗原 G5 的基因修饰猪的构建. 生物工程学报, 2022, 38(3): 1096-1111.
ZHOU XQ, LIU Y, TANG CC, CHENG LY, ZHENG SW, ZHENG YL, CHEN M, YANG HQ, ZOU QJ, LAI LX. Generation of genetically modified pigs devoid of GGTA1 and expressing the human leukocyte antigen-G5. Chin J Biotech, 2022, 38(3): 1096-1111.

摘 要：由于猪的诸多生理和遗传特征与人类近似，猪一直被认为是理想的异种器官移植候选供体动物。但是物种之间严重的免疫不相容是影响猪到灵长类异种器官移植的最关键因素。为了克服物种之间的免疫排斥反应，诸多研究对猪进行了各种各样的基因修饰，消除或抑制造成异种器官移植的免疫原，以获得可应用于灵长类移植的供体器官。本研究利用转录激活因子样效应物核酸酶 (transcription activator-like effector nuclease, TALEN) 对猪的 α-1,3-半乳糖苷转移酶 (α-1,3-galactosyltransferase, GGTA1) 基因进行敲除，该基因编码的酶催化合成 α-1,3-半乳糖，这是导致猪到灵长类异种器官移植中产生超急性免疫排斥的关键因子。同时本研究也通过转基因方法导入一种重要的免疫抑制因子，人类白细胞抗原 G5 (human leukocyte antigen-G5, HLA-G5)，来进一步增加猪器官在异种器官移植中的免疫耐受。本研究筛选了 *GGTA1* 双等位基因敲除 (*GGTA1* knockout, GTKO) 且 *HLA-G5* 转基因导入的猪成纤维细胞，并通过体细胞核移植 (somatic cell

Received: August 28, 2021; **Accepted:** November 12, 2021
Supported by: National Natural Science Foundation of China (81941001); Natural Science Foundation of Guangdong Province, China (2019A1515110283); Science and Technology Planning Project of Jiangmen, Guangdong Province, China (2021030101220004887); Science Foundation for Young Faculty of Wuyi University (2019TD05)
Corresponding author: LAI Liangxue. E-mail: lai_liangxue@gibh.ac.cn
#These authors contributed equally to this study
基金项目：国家自然科学基金 (81941001)；广东省自然科学基金 (2019A1515110283)；江门市科技计划 (2021030101220004887)；五邑大学青年人才基金 (2019TD05)

nuclear transfer, SCNT) 构建相应的基因修饰猪模型。本研究获得了 20 头 *GGTA1* 双等位基因敲除的克隆猪，其中 10 头同时表达 HLA-G5 蛋白。经检测，α-1,3-半乳糖在 GTKO/HLA-G5 克隆猪的组织和细胞上完全缺失，Western blotting 和免疫荧光染色显示，HLA-G5 在克隆猪的组织和细胞上成功表达。功能学分析显示，从 GTKO/HLA-G5 克隆猪分离的成纤维细胞具有更好的免疫耐受，相比于单一的 GTKO 猪和未经基因修饰的野生型猪，GTKO/HLA-G5 克隆猪的细胞对补体介导的细胞裂解反应具有更强的耐受。GTKO/HLA-G5 克隆猪可以为异种器官移植提供一种有用的动物模型。

关键词：基因修饰猪；GGTA1；基因敲除；HLA-G5；异种器官移植；TALEN

Generation of genetically modified pigs devoid of GGTA1 and expressing the human leukocyte antigen-G5

ZHOU Xiaoqing[1,2#], LIU Yu[1#], TANG Chengcheng[1,2], CHENG Lingyin[1], ZHENG Shuwen[1], ZHENG Yuling[1], CHEN Min[1,2], YANG Huaqiang[3], ZOU Qingjian[1,2], LAI Liangxue[1,4]

1 School of Biotechnology and Health Science, Wuyi University, Jiangmen 529020, Guangdong, China
2 Guangdong Provincial Key Laboratory of Large Animal Models for Biomedicine, Jiangmen 529020, Guangdong, China
3 College of Animal Science, South China Agricultural University, Guangzhou 510642, Guangdong, China
4 Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, Guangdong, China

Abstract: Pigs are considered as ideal donors for xenotransplantation because they have many physiological and anatomical characteristics similar to human beings. However, antibody-mediated immunity, which includes both natural and induced antibody responses, is a major challenge for the success of pig-to-primate xenotransplantation. Various genetic modification methods help to tailor pigs to be appropriate donors for xenotransplantation. In this study, we applied transcription activator-like effector nuclease (TALEN) to knock out the porcine α-1,3-galactosyltransferase gene *GGTA1*, which encodes Gal epitopes that induce hyperacute immune rejection in pig-to-human xenotransplantation. Meanwhile, human leukocyte antigen-G5 gene *HLA-G5*, which acts as an immunosuppressive factor, was co-transfected with TALEN into porcine fetal fibroblasts. The cell colonies of *GGTA1* biallelic knockout with positive transgene for *HLA-G5* were chosen as nuclear donors to generate genetic modified piglets through a single round of somatic cell nuclear transfer. As a result, we successfully obtained 20 modified piglets that were positive for *GGTA1* knockout (GTKO) and half of them expressed the HLA-G5 protein. Gal epitopes on the cell membrane of GTKO/HLA-G5 piglets were completely absent. Western blotting and immunofluorescence showed that HLA-G5 was expressed in the modified piglets. Functionally, the fibroblasts from the GTKO/HLA-G5 piglets showed enhanced resistance to complement-mediated lysis ability compared with those from GTKO-only or wild-type pigs. These results indicate that the GTKO/HLA-G5 pigs could be a valuable donor model to facilitate

laboratory studies and clinics for xenotransplantation.

Keywords: gene-modified pig; GGTA1; gene knockout; HLA-G5; xenotransplantation; TALEN

Pigs are considered as appropriate donor organisms for xenotransplantation because they have many similar physiological and anatomical characteristics compared with human beings. The plentiful sources and low cost also make them a suitable choice. However, the greatest challenge of pig-to-primate xenotransplantation is the cascade rejection, which includes hyperacute rejection (HAR), acute cellular rejection, delayed xenograft rejection, and chronic rejection[1]. Among these immune rejections, HAR is the primary and key problem that needs to be addressed. Gal-α1,3-Gal, the main cause of HAR, is an oligosaccharide that is produced on the cell surface of pigs, but not in human beings, apes, or old world monkeys[2]. Disruption of α-1,3-galactosyltransferase (GGTA1), the pivotal enzyme that synthesizes Gal-α1,3-Gal, is essential to overcome HAR. Kolber-Simonds et al. firstly produced pigs with GGTA1 disrupted for experimental studies[3]. Many studies have shown that the frequency of hyperacute rejection was decreased in the transplantation of organs from *GGTA1*-knockout (GTKO) pigs into nonhuman primates[4-5]. Further reduction of xenotransplant rejection may require more modifications in the GTKO genetic background to overcome the complex immunobiological barriers, including transgenesis with specific genes, singly or in combination, to inhibit complement activation, cellular rejection, or dysregulation of coagulation and inflammation, thereby improving the long-term survival of porcine xenografts after transplantation. To date, some multi-transgenic GTKO pigs, e.g., those with knock-in five human genes [$GT^{-(DAF/CD39/TFPI/C1\text{-}INH/TNFAIP3)/+}$], have been generated[6]. These pig models are expected to further attenuate immune rejection and physiological incompatibilities in pig-to-primate transplantation.

Human leukocyte antigen-G (HLA-G) belongs to the non-classical class-Ⅰ molecule family, and plays a particular role during pregnancy. The expression of HLA-G at the maternal-fetal barrier facilitates the immune tolerance of the allogenic fetus[7-8]. According to reports, besides restricted expression at the maternal-fetal barrier, HLA-G is also expressed in some transplanted patients, demonstrating its role in the regulation of allogenic response[9-11]. Unlike classical class-Ⅰ molecule, HLA-G has seven different isoforms, among which HLA-G1, -G2, -G3 and -G4 bind to the cell membrane, whereas HLA-G5, -G6 and -G7 are dissolved in plasma. Functionally, HLA-G inhibits the ongoing proliferation of T cell and NK cells[12-13], and the cytolytic activity of T lymphocytes[14-16]. HLA-G does inhibit immune responses by interacting with inhibitory receptors, which are expressed from antigen-presenting cells and lymphocytes[17-18]. Three HLA-G receptors have currently been identified. ILT2 (CD85j/LILRB1) is expressed by some T cells, some NK cells, all B cells and monocytes/dendritic cells[19]. ILT4 (CD85d/LILRB2) is expressed by myeloid cells[20]. KIR2DL4 (CD158d) is expressed by some NK cells and peripheral cells[21]. The various ligand-receptor interactions, and the restricted expression at the feto–maternal barrier or in some transplanted patients, both demonstrated that HLA-G had a significant effect on the regulation of the allogenic response.

In general, the HLA-G molecules that we usually mention refer to HLA-G1 (membrane-bound isoform) and HLA-G5 (soluble isoform)[22-23]. The level of soluble HLA-G after allogeneic transplantation is associated with better transplantation function. Among some kidney transplant patients, the incidence of acute rejection was significantly reduced in patients with elevated plasma HLA-G levels[9]. In addition,

in some heart transplant patients, soluble HLA-G levels were inversely related to organ failure caused by humoral rejection, which indicated its inhibition of immune rejection[10]. These reports all demonstrated that HLA-G is associated with a lower risk of acute and chronic rejection in allogeneic transplantation.

In this study, we tried to take advantage of the immunosuppressing potential of HLA-G to confer enhanced tissue compatibility of gene-modified pig organs in xenotransplantation. We used transcription activator-like effector nuclease (TALEN)-mediated genetic modification and somatic cell nuclear transfer (SCNT) to generate GTKO pigs expressing HLA-G5 to establish more appropriate donors for xenotransplantation.

1 Materials and methods

1.1 Animals

All works were performed in accordance with Public Health Service Policies, the Animal Welfare Act, and the Laboratory Animal Committee (LAC) of GIBH Policy on the Humane Care and Use of Vertebrate Animals (No. X12021040).

1.2 The TALENs of *GGTA1* and the HLA-G5-expressing vectors

The TALENs of *GGTA1* were purchased from ViewSolid Biotech (Beijing, China). The *GGTA1* target site is 5′-CTTATCCCCAGAATAC TGCTGGGATTATCATATAGGCATGTCTGTGGA T-3′, which is located in exon 6 of the porcine *GGTA1* gene[24]. The *HLA-G5* gene was obtained from the Chinese PLA general hospital and cloned downstream of the CAG promoter for overexpression. A neomycin-resistant gene was included in the HLA-G5-expressing vector as a selective marker to generate transgenic cells.

1.3 Cell transfection and colony selection

Bama porcine fetal fibroblasts (PFFs) were isolated from 35-day-old male Bama fetuses and digested by collagenase IV-DNase in cell culture medium containing 0.32 mg/mL collagenase IV and 2 500 IU/mL DNase for 4 to 6 h at 39 °C. The PFFs were cultured in 10 cm dishes for 12 h and frozen in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide for later use. One day before the transfection, PFFs were removed from liquid nitrogen, then quickly thawed in water at 37 °C and transferred to a 15 mL centrifuge tube. Cell culture media (DMEM with 10% FBS) were slowly added with gently shaking and mixing. Centrifugation was performed for 5 min at 300× *g*/min. Then the supernatant was carefully removed and 2 mL of medium was added. All precipitate (PFF cells) were transferred into a 10 cm dish, to which 8 mL of medium was added. Then they were cultured in the cell culture incubator at 38 °C. When the cells recovered in the previous day have reached approximately 90% of the 10 cm dish, approximately 10^6 PFFs were electroporated with the TALENs of *GGTA1* and HLA-G5-expressing vector at 230 μV and 500 μF using a Gene Pulse Xcell electroporator (Bio-Rad, USA). After electroporation, all cells were inoculated into a new 10 cm dish. After 24 h incubation, 1 mg/mL of G418 was added to start and maintain the selection for 10 d. When the cell colonies appeared, all the colonies were placed into 48-well plates for cultivation, and one fifth of each colony cell was obtained for PCR identification. The PCR primers were listed in supplementary Table 1. All PCR products were subjected to Sanger sequencing (BGI sequencing) to detect the presence of insertions and/or deletions (indels) induced by TALENs. The positive cell colonies with both *GGTA1* knockout and *HLA-G5* positive were passaged to 24-well plates for expanded culture.

Table 1 The efficiency of TALEN-mediated *GGTA1* gene-targeting in PFFs

Target gene	No. screen	No. (Percent of mono-knockout)	No. (Percent of double-knockout)	No. (Percent of knockout)

GGTA1	124	48 (38.7%)	22 (17.7%)	70 (56.4%)

1.4 Pig cloning

Methods used for pig cloning, including porcine oocyte collection, *in vitro* maturation, SCNT, and embryo transfer were similar to those used in our previous studies[25-26]. Briefly, the dense and homogeneous cumulus oocyte complexes (COCs) were cultured in a mature medium at 39 °C for 42–44 h. The first polar bodies were aspirated to obtain enucleated oocytes, then the selected donor cells (positive cell colonies) were injected into the enucleated oocytes to obtain reconstructed embryos. All the reconstructed embryos were fused and activated by an electrofusion instrument. Then all the reconstructed embryos were cultured at 39 °C overnight and taken out for embryo transfer. Through surgery, embryos were implanted deep into the fallopian tubes, and at least 200 reconstructed embryos were transferred into each pig. After one month, the surrogate sows who did not return to the sex were subjected to the first ultrasonic pregnancy test. After the pregnancy was detected, the surrogate sow was regularly tested once a week and monitored until two weeks before the expected date of delivery. After a gestation period of about 114 d, cloned pigs were obtained through natural delivery. The genotypes of the cloned piglets were identified by the same methods shown in the “cell transfection and colony selection” section by using the genomic DNA extracted from ear biopsies.

1.5 Western blotting

The ear fibroblasts and some other tissues from cloned piglets were lysed in RIPA buffer and then boiled in SDS sample buffer at 100 °C for 5 min. SDS-PAGE was used to separate different proteins in the lysate and then a PVDF membrane was used to transferred all proteins by electrophoresis. To detect HLA-G5, the membrane and anti-HLA-G antibody (Abcam, USA) were incubated overnight at 4 °C. After three times wash, the goat anti-mouse IgG secondary antibody was incubated with the membrane at room temperature for approximately 1 h. After three times wash, the SuperSignal West PicoTrial enhanced chemiluminescence kit (Thermo, USA) was used to detect HLA-G5 levels. β-actin was detected as load control. Secondary antibody was goat anti-mouse IgG (bs-0368G-HRP, Boiss, China). β-actin antibody was RG000120 (Solarbio, China).

1.6 Flow cytometric analysis

Both WT and GTKO/HLA-G5 fibroblasts were stained with FITC-GS-IB4 lectin (L2895, Sigma, USA) to detect Gal-α1,3-Gal epitope expressions. The harvested cells were resuspended and washed three times in PBS, and then treated with 20 μg/mL FITC-GS-IB4 lectin at 37 °C for 5 min. All cells were resuspended quickly in 300 μL PBS and then immediately detected by BD Accuri C6 flow cytometry to calculate the ratio of gene-knockout cells in the cell clones.

1.7 Immunofluorescences (IF) and immunohistochemistry (IHC)

IF and IHC were conducted in cultured fibroblasts or sectioned tissues to stain the GGTA1 or HLA-G5 levels. For cell staining, both WT and GTKO/HLA-G5 fibroblasts were grown in 24-well plates in the same density for 24 h. Then, all cells were fixed with 4% paraformaldehyde for 10 min. For tissues, heart, lung, spleen and kidney from GTKO/HLA-G5 pigs were fixed with 4% paraformaldehyde for 2 d. Then, various tissues were sliced into a thickness of approximately 3 μm. After dewaxing and dehydration, the slices were subjected to antigen recovery in a 1 mol/L citrate buffer for about 20 min. Afterwards, all cells and tissue sections were blocked with 5% BSA for 1 h. After three times wash, the samples were incubated with the αGal epitope monoclonal antibody (ALX-801-090-1, Enzo Life Sciences, Lausen, Switzerland) or anti-HLA-G antibody overnight at 4 °C. After three times wash, the samples were

incubated for 2 h with FITC-conjugated secondary antibodies (for immunofluorescences) or VECTASTAIN ABC kits (peroxidase) (Vector Laboratories, USA) (for immunohistochemistry).

1.8 Immunophenotyping of blood lymphocytes

Anticoagulated blood from GTKO/HLA-G, GTKO-only, and wild-type (WT) pigs were collected. Red blood cells were lysed, and mononuclear cells were stained with antibodies specific for pig/human CD56 (304604, San Diego, USA), porcine IgM (AAI39F; AbD Serotec, UK), and porcine CD4a (559585, BD Biosciences, USA)/CD8a (559584; BD Biosciences, USA) to label NK, B, and T cells, respectively. An AccuriC6 flow cytometer (BD, USA) was used to collect and analyze the data. A population of more than 10 000 live cells was analyzed per sample.

1.9 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

MTT assay was used to investigate the sensitivity of modified cells from cloned piglets to cell lysis caused by human complement system. Porcine fibroblasts (2×10^5) from cloned piglets were incubated in PBS for about 6 h with 10% normal human serum (The author volunteered to donate 15 mL own blood, which was then precipitated and taken out the upper serum). All treated cells were transferred to 96-well plates and then cultured in DMEM of 15% FBS for 8 h. According to the manufacturer's protocol, cell viability was assessed using the MTT cell proliferation and cytotoxicity assay kit (Beyotime, China). The accumulation of soluble formazan was detected at 490 nm by a spectrophotometer. The cell survival was calculated by the following formula, living cells (%) = absorptivity of normal human serum-treated cells/absorptivity of untreated cells.

2 Results and Analysis

2.1 Generation of GTKO/HLA-G5 porcine fibroblasts

The plasmids of TALEN pair targeting the exon 6 of porcine *GGTA1* gene were commercially constructed and the structures of TALENs are shown in Figure 1A. We also constructed a HLA-G5-expressing vector that harbors a neomycin resistant gene as selective marker (Figure 1B). To simultaneously target *GGTA1* and overexpress *HLA-G5* transgene, we co-transfected the TALENs of HLA-G5-expressing vector into Bama porcine fetal fibroblasts by electroporation. All cell colonies were incubated with 1 mg/mL of G418 for approximately 10 d. Then, living colonies were picked for PCR identification of TALEN-targeting region of *GGTA1*. Our results showed that the frequency of GTKO modification was 56.4% (70/124) and the rate of *GGTA1* double-knockout was 17.7% (22/124) in all genotyped colonies (Table 1).

2.2 Generation of GTKO/HLA-G5 genetic modified piglets via SCNT

Seven GTKO/HLA-G5-positive cell colonies, containing indels from 4 to 163 bp, were chosen as donors for SCNT. Among the seven cell colonies, C10, C18, and C40 were positive for *HLA-G5* transgene; C7, C56, C61 and C66 were *HLA-G5* negative as for only GTKO control (Table 2). Three to four colonies were usually pooled in one nuclear transfer to produce reconstructed embryos in order to avoid the poor quality of one clone. We introduced 1 977 reconstructed embryos from GTKO/HLA-G5 cells into 12 appropriate surrogates. Among the 12 surrogates, seven became pregnant and finally gave birth to 20 male cloned piglets (Table 3). Compared with WT pigs, GTKO/HLA-G5 pigs were similar in growth and size (Figure 1C). PCR and Sanger sequencing analysis results demonstrated that the 20 piglets were all homozygous for GTKO, and half of which (10/20) were positive for the *HLA-G5* transgene (Table 4 and Figure 1D). Among the 20 piglets, nine piglets were born weak and died within 2 weeks. Five piglets were healthy and normal at birth but also died within a

month possibly duo to unidentified infections. The remaining six pigs developed normally, but died of African swine fever after one year (Table 5).

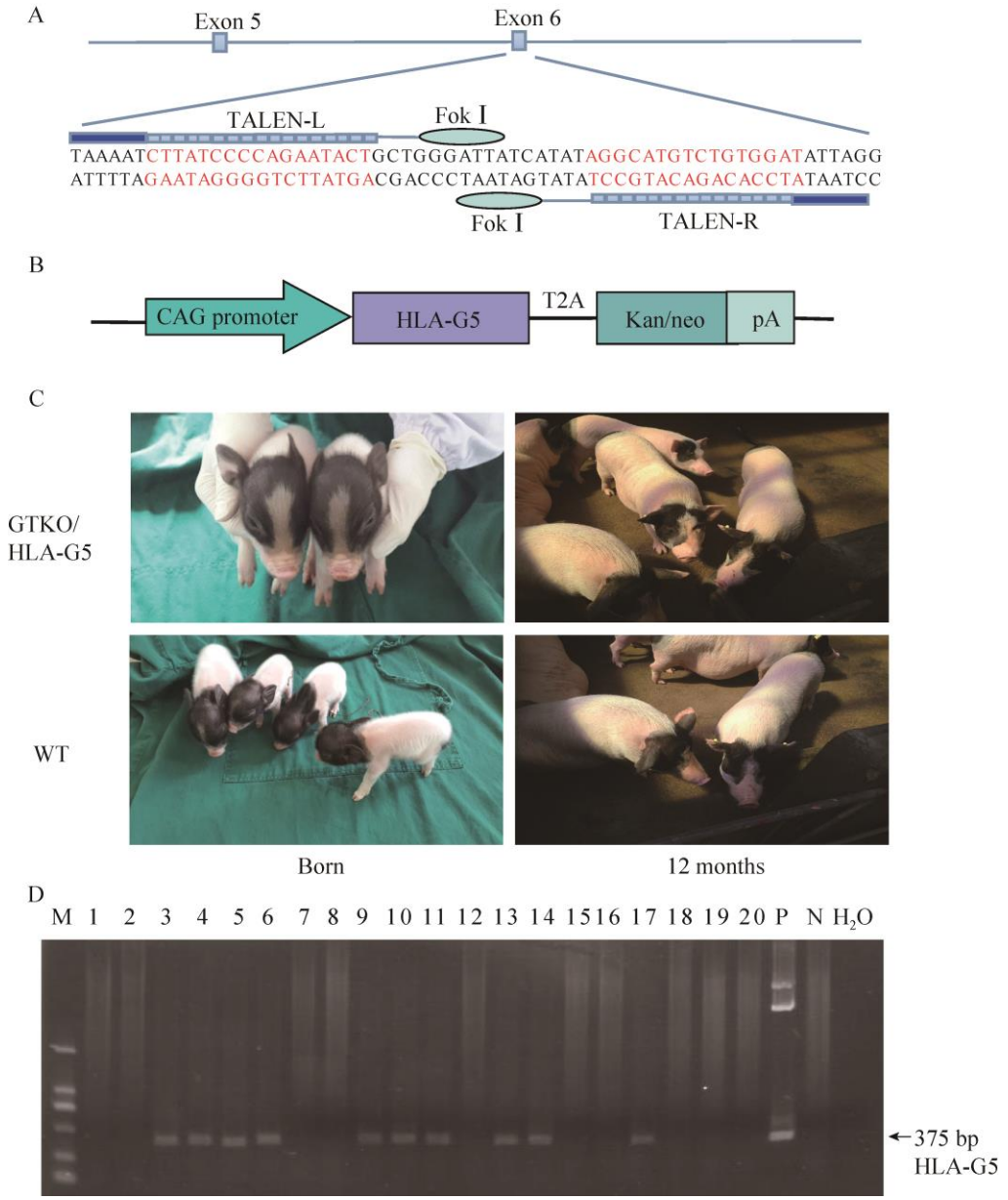

Figure 1 Generation of genetic modified piglets via SCNT. (A) Schematic of TALENs targeting *GGTA1* loci. The target loci are located in the sixth exon. TALENs targeting sites are highlighted in red. (B) Structure of HLA-G5-expressing vector. (C) The pictures of GTKO/HLA-G5-gene-modified pigs. The left panel shows two newborn GTKO/HLA-G5 piglets and four age-matched wild-type piglets. The right panel shows two 12-month-old GTKO/HLA-G5 pigs and two age-matched wild-type (WT) pigs. (D) PCR results of *HLA-G5* in cloned pigs. M is the 2 000 bp marker. P is HLA-G5-expressing vector as a positive control. N is WT cell as a negative control.

2.3 Phenotypic characterization of GTKO/HLA-G5 pigs

We examined whether gene alterations caused desired phenotypes in the cloned genetically modified pigs. Isolectin B4 has a high affinity for α-galactose epitope (αGal). Therefore, we used FITC-GS-IB4 lectin to detect the αGal of GTKO porcine cells. By staining the ear fibroblasts of the modified piglets with FITC-GS-IB4 lectin and observation under fluorescence microscope, we found that the cells of GTKO/HLA-G5 piglets were completely absent for lectin staining (Figure 2A). The same results were obtained by assay with flow cytometry, which showed that no FITC-positive cells were present in isolated fibroblasts of GTKO/HLA-G5 piglets, whereas WT fibroblasts were all positive (Figure 2B). We further evaluated the Gal-α1,3-Gal levels in the cells and tissues of GTKO/HLA-G5 piglets with an anti-αGal antibody. IF assay results confirmed that the ear fibroblasts of the GTKO/HLA-G5 piglets were αGal negative on their surface (Figure 2C). Histological sections of various tissues assayed with anti-αGal antibody staining showed that the expression of αGal could not be recognized in the organs of GTKO/HLA-G5 piglets, including heart, liver, spleen, and lung, whereas all the tested tissues from WT piglets showed evident αGal expression (Figure 2D). These results collectively proved that Gal-α1,3-Gal-negative pigs with both *GGTA1* alleles inactivated were obtained.

Table 2 Sanger sequencing of the targeting sites of *GGTA1* in mutant colonies used in SCNT

Colony number	*GGTA1* genotypes	*GGTA1* indels	*HLA-G5*
	AGAATACTGCTGGGATTATCATATAGGCA	WT	–
C7	AGAATACTGCTGG--------ATCATATAGGCA	△4/△13/△14	–
	AGAATACTGCTG----------CA		
	AGAATACTGCTGGG----------		
C10	AGAATACTGCTGGGATTATtaCATATAGGCA	+2/+2	+
C18	AGAATACTGCTG----------	△132/△132	+
C40	AGAATACTGCTG----------	△163/△163	+
C56	AGAATACTGCTGG---------ATCATATAGGCA	△4/△5	–
	AGAATACTGCTGG----------TCATATAGGCA		
C61	AGAATACTGCTG----------AGGCA	△9/△13	–
	AGAATACTGCTG----------CA		
C66	AGAATACTGCTG----------	△44 /△44	–

The WT sequence is shown at the top with the target sites highlighted in bold. The net change in length caused by each mutation is to the right of each sequence (+, insertion; Δ, deletion). The presence (+) or absence (–) of transgene *HLA-G5* in each colony is also shown.

Table 3 Summary of SCNT results for the generation of GGTA1/HLA-G pigs

Target gene	Cell clones	Reconstructed embryos	No. recipients	No. (Percent of pregnancies)	No. born	No. (Percent of *GGTA1* mutant)	No. (Percent of *HLA-G5* transgene)
GGTA1/ *HLA-G5*	C7, C18, C40	256	2	2	5	5	3
	C10, C18, C40	329	2	1	4	4	2
	C40, C56, C61	405	2	0	0	0	0
	C18, C40, C56	316	2	1	3	3	2
	C10, C61, C66	329	2	1	4	4	2

	C18, C56, C61, C66	342	2	2	4	4	1
Total	7	1 977	12	7 (58.3%)	20	20 (100%)	10 (50%)

Table 4 Validation of *GGTA1* and *HLA-G5* at the genomic level in all cloned pigs

Piglet number	*GGTA1* genotypes	*GGTA1* indels	Amino acids sequence	*HLA-G5*
	TTATCCCCAGAATACTGCTGGGATTATCATATAGGCATGTCTGTG	WT	LSPEYCWDYHIGMSV	–
G1	TTATCCCCAGAATACTGCTG----------CATGTCTGTG TTATCCCCAGAATACTGCTGGG----------TGTCTGTG	△13/△14	LSPEYCCMSV LSPEYCWVSV	–
G2	TTATCCCCAGAATACTGCTGGGATTATtatCATATAGGCATGTCTGTG	+3/+3	LSPEYCWDYYHIGMSV	–
G3, G4, G9, G11	TTATCCCCAGAATACTGCTG----------	△163/△163	LSPEYC	+
G5, G10, G17	TTATCCCCAGAATACTGCTG----------	△132/△132	LSPEYC	+
G6, G13, G14	TTATCCCCAGAATACTGCTGGGATTATtaCATATAGGCATGTCTGTG	+2/+2	LSPEYCWDYYI*	+
G7, G8, G16	TTATCCCCAGAATACTGC----------ATCATATAGGCATGTCTGTG TTATCCCCAGAATACTGC----GGGATTATCATATAGGCATGTCTGTG	△1/△7	LSPEYCII* LSPEYCGIII*	–
G12	TTATCCCCAGAATACTGCTGG---------ATCATATAGGCATGTCTGTG TTATCCCCAGAATACTGCTGG----------TCATATAGGCATGTCTGTG	△4/△5	LSPEYCWII*	–
G15, G20	TTATCCCCAGAATACTGCTG----------AGGCATGTCTGTG TTATCCCCAGAATACTGCTG----------CATGTCTGTG	△9/△13	LSPEYCWSYRHVC LSPEYCCMSV	–
G18, G19	TTATCCCCAGAATACTGCTG----------	△44 /△44	LSPEYC	–

For *GGTA1*, the mutant alleles were tested by Sanger sequencing. WT sequence is shown at the top in which the target sites are highlighted in bold. The next column to the right contains the indel size of each allele. Deletion and insertion are denoted as "Δ" and "+" plus the number of base pairs. For *HLA-G5* gene, positive and negative are denoted as "+" and "–". Terminator is denoted as *.

We next performed Western blotting to analyze HLA-G5 expression in cultured fibroblasts and multiple organs (heart, liver, spleen, lung, and kidney) from the GTKO/HLA-G5 pigs and age- and breed-matched WT pig. GTKO/HLA-G5 pigs clearly showed the presence of HLA-G5 protein in their fibroblasts and various tissues (Figure 3A). IF analysis revealed HLA-G5 protein was successfully expressed in the fibroblasts from GTKO/HLA-G5 pigs (Figure 3B), and HLA-G5 protein were also existed in the organs (heart, spleen, lung, and kidney) of the genetically modified pigs compared with the non-existence of HLA-G5 in the WT control (Figure 3C).

We further characterized the changes in lymphocyte subsets of GTKO/HLA-G5 pigs compared with WT and GTKO-only pigs. The T cell, B cell, and NK cell populations in peripheral blood were stained with CD4/CD8, IgM and CD56 antibodies, respectively, and analyzed by flow cytometry. This analysis revealed that $CD56^+$ NK cells in GTKO and GTKO/HLA-G5

pigs were significantly lower than those in WT pigs, and did not differ in GTKO and GTKO/HLA-G5 pigs. IgM^+ B cells were also lower in GTKO and GTKO/HLA-G5 pigs than WT pigs, and GTKO/HLA-G5 pig exhibited further reduced IgM^+ B cells compared with GTKO pig. However, $CD4^+/CD8^+$ T cells were greatly increased in the modified pigs, and an equal $CD4^+/CD8^+$ T cell level was found between GTKO/HLA-G5 and GTKO pigs (Figure 4). These results suggested that either GTKO or *HLA-G5* transgenes can interfere with lymphocyte differentiation in transgenic pigs.

Table 5 Summary of genetically modified pigs for their health status and origin donor cells

Piglet ID	Gestation length (d)	Birth weight (g)	Health status	Donor cell clone	Donor cell clone poll
G1	119	1 850	Survive for 25 days	C7	C7, C18, C40
G2	119	2 850	Survive for 1 year	ND*	C7, C18, C40
G3	119	2 300	Survive for 22 days	C40	C7, C18, C40
G4	119	1 750	Survive for 1 year	C40	C7, C18, C40
G5	119	1 200	Survive for 1 day	C18	C7, C18, C40
G6	118	900	Survive for 1 day	C10	C10, C18, C40
G7	118	850	Survive for 1 day	ND*	C10, C18, C40
G8	118	650	Survive for 1 year	ND*	C10, C18, C40
G9	118	950	Survive for 30 days	C40	C10, C18, C40
G10	120	500	Survive for 1 day	C18	C18, C40, C56
G11	120	500	Survive for 1 year	C40	C18, C40, C56
G12	120	550	Survive for 1 day	C56	C18, C40, C56
G13	122	550	Survive for 1 day	C10	C10, C61, C66
G14	122	600	Survive for 1 year	C10	C10, C61, C66
G15	122	600	Survive for 1 year	C61	C10, C61, C66
G16	122	500	Survive for 15 days	ND*	C10, C61, C66
G17	120	200	Survive for 1 day	C18	C18, C56, C61, C66
G18	120	250	Survive for 2 days	C66	C18, C56, C61, C66
G19	120	300	Survive for 2 days	C66	C18, C56, C61, C66
G20	120	300	Survive for 19 days	C61	C18, C56, C61, C66

ND: not determined. *: these donor cells may account for a very low percentage in some colonies, thus are not able to be distinguished through sequencing.

2.4 Enhanced resistance to complement-mediated lysis ability of GTKO/HLA-G5 fibroblasts

The sensitivity of fibroblasts from GTKO/HLA-G5 piglet to complement-mediated lysis was tested using MTT analysis with complete human serum. We selected ear fibroblasts from three GTKO/HLA-G5 piglets (numbers G3, G9 and G14) and three GTKO-only piglets (numbers G1, G2 and G20). All these ear fibroblasts were derived from piglets when they were healthy. MTT analysis experiments were repeated thrice, and the results differed significantly. As shown in Figure 5, approximately 73.2% (the average

value of three pigs) of the fibroblasts from the GTKO/HLA-G5 pigs were survived after complement-mediated lysis, 65.3% of the fibroblasts from the GTKO pigs were survived, whereas only approximately 22.7% of the fibroblasts from WT pigs survived. The cell viability rate of GTKO/HLA-G5 pigs was higher than that of GTKO pigs, suggesting that fibroblasts from the GTKO/HLA-G5 pigs showed enhanced resistance to complement-mediated lysis ability compared with fibroblasts from WT pigs and GTKO-only pigs.

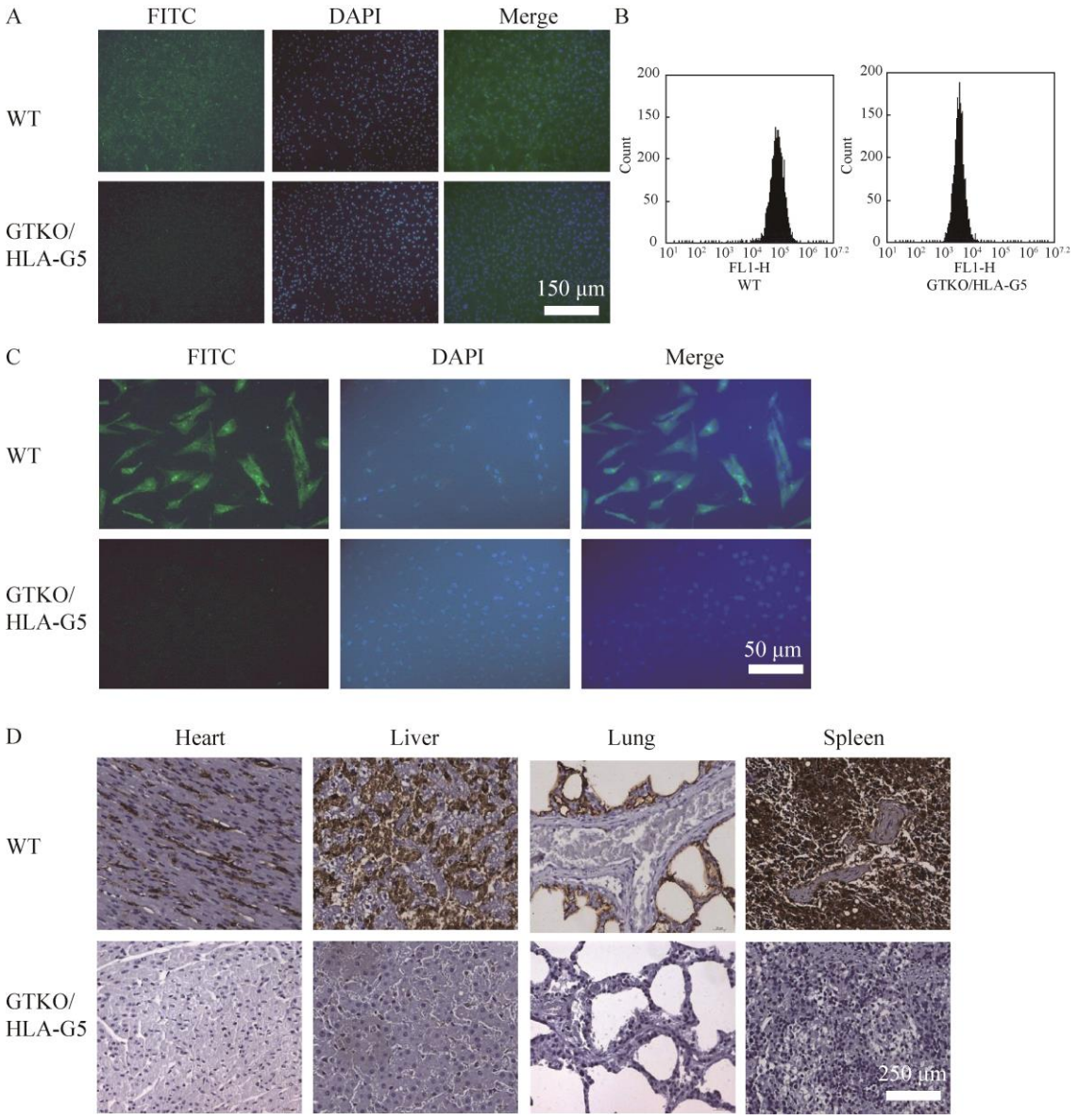

Figure 2 GGTA1 expression of GTKO/HLA-G5 piglets. (A) Porcine ear fibroblasts stained with

FITC-conjugated GS-IB4 were observed under fluorescence microscopy. The fibroblasts of wild-type (WT) pig were used as the positive control. (B) Fibroblasts from the cloned pigs were analyzed by FACS with FITC-conjugated GS-IB4 lectin staining. GTKO/HLA-G5 pigs were proven to be α 1,3-Gal-negative. (C) IF assay with antibody. Fibroblasts from cloned pigs were stained with the αGal epitope monoclonal antibody. The fibroblasts of WT pig were used as the positive control. (D) IHC assay of pig tissues with antibody. The major tissues were stained with the αGal epitope monoclonal antibody. GTKO/HLA-G5 pig is G3, WT pig is an age-matched wild-type Bama mini-pig.

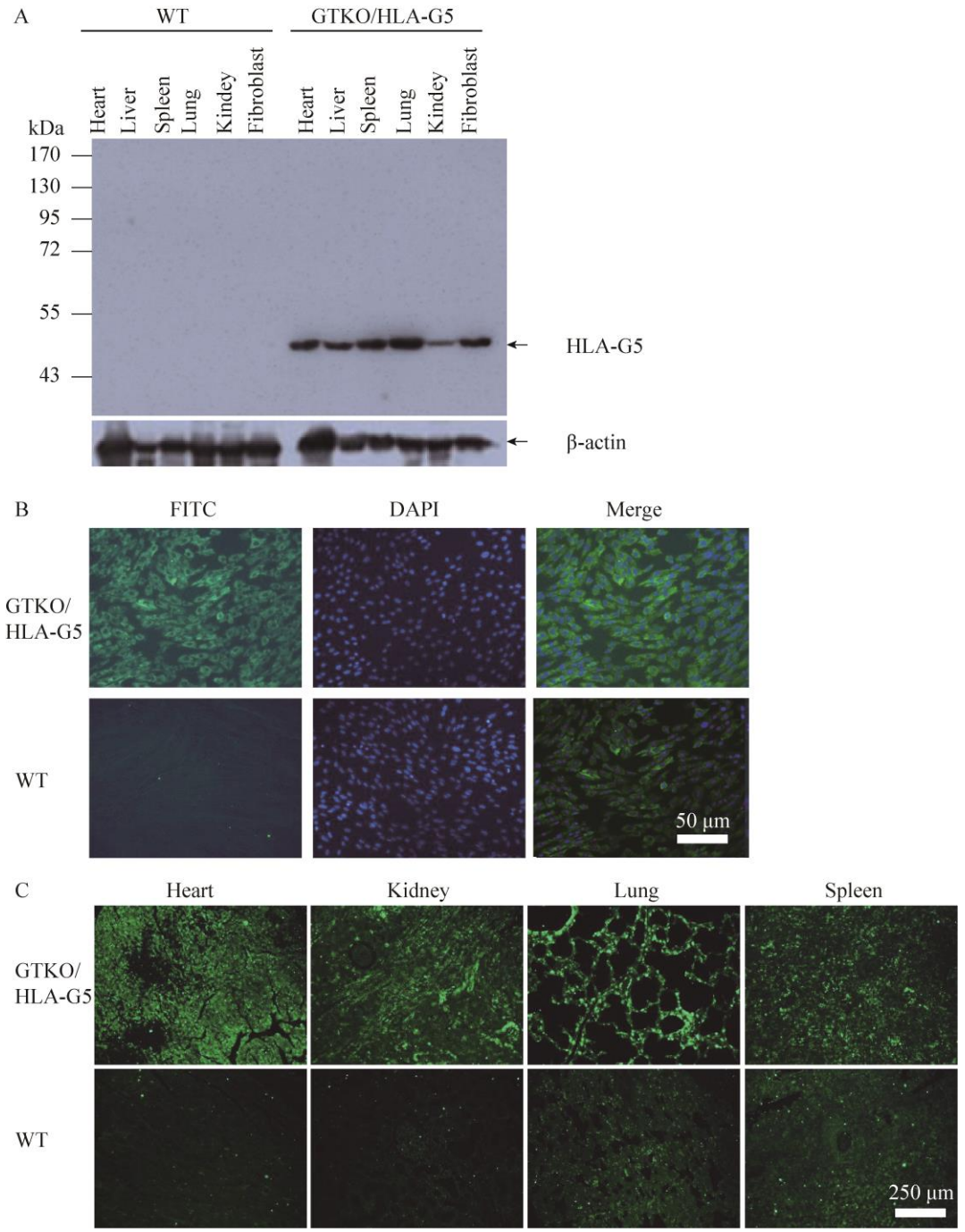

Figure 3 HLA-G expression of GTKO/HLA-G5 piglets. (A) Western blotting analysis of tissues and cells of GTKO/HLA-G5 and wild-type (WT) pigs. Significant HLA-G5 expression is seen in GTKO/HLA-G5 pig but not in WT pigs. β-actin was used as the internal control. (B) IF assay for HLA-G5 in isolated fibroblasts. Fibroblasts from GTKO/HLA-G5 pigs were stained with anti-HLA-G antibody. The fibroblasts of WT pig were used as negative control. GTKO/HLA-G5 pig is G3, WT pig is an age-matched wild-type Bama mini-pig. (C) HLA-G5 IF assay of pig tissues. The major tissues of modified and WT pigs were stained with anti-HLA-G antibody.

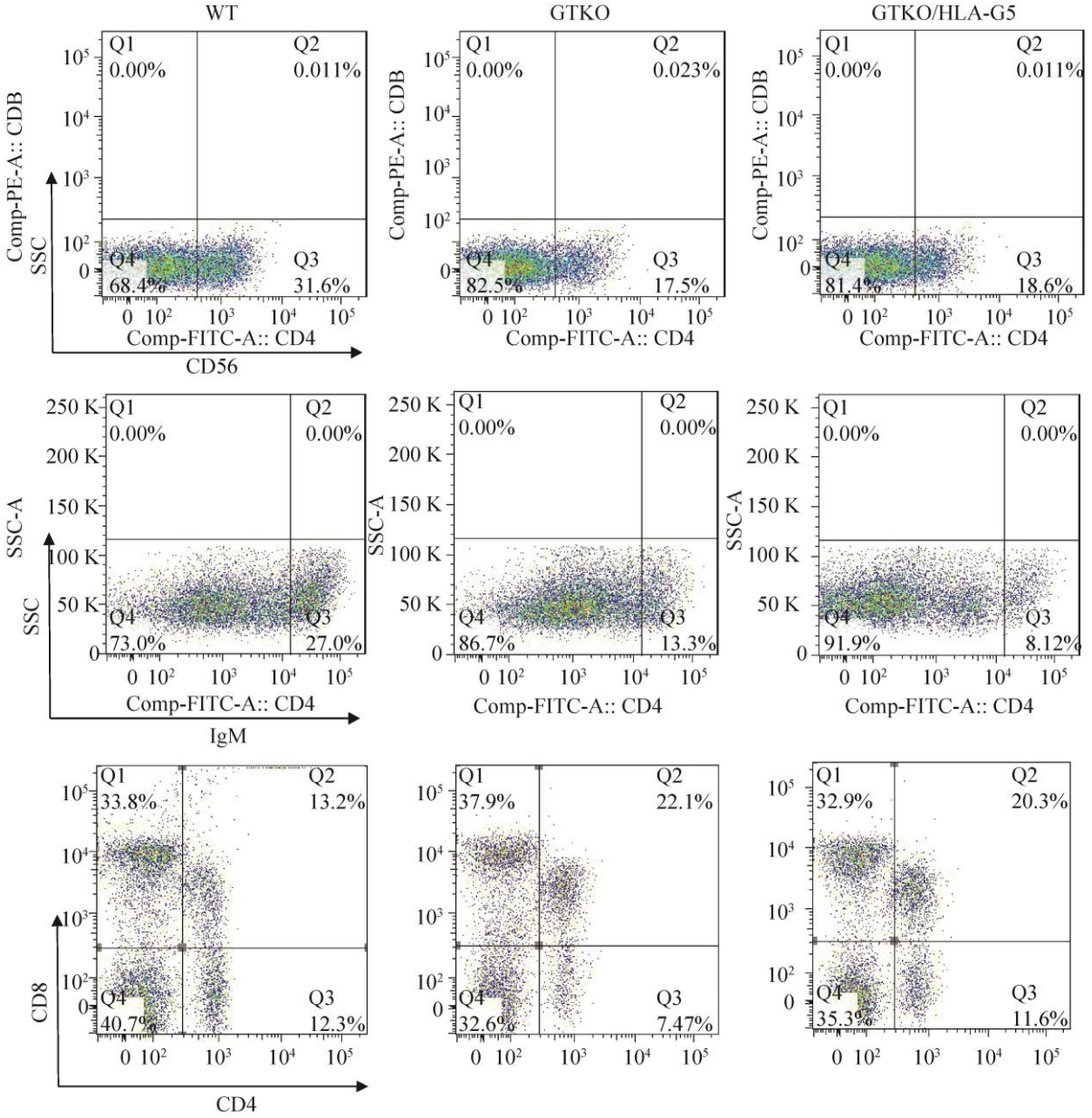

Figure 4 FACS analysis of B lymphocytes, T lymphocytes and NK cells from the peripheral blood of GTKO/HLA-G5, GTKO-only, and wild-type (WT) pigs. The cells were stained with antibodies of CD4/CD8, IgM and CD56 to label T lymphocytes, B lymphocytes and NK cells, respectively. GTKO/HLA-G5 pig is G3,

GTKO-only pig is G1, and WT pig is an age-matched wild-type Bama mini-pig.

3 Discussion

The clinical application of xenotransplantation depends on the establishment of genetically modified pigs whose organs are not easily rejected by the human immune system. The disruption of *GGTA1* gene is the first and important step to overcome HAR. Many strategies including various transgenes which were able to suppress the immune rejection from the recipients were used to modify the GTKO pigs to further increase the immune tolerance of donor organs in xenotransplantation. For example, when organs were transplanted from pigs expressing one or more human complement regulatory proteins, such as CD46, CD55, or CD59, even from GTKO pigs expressing human complement regulatory proteins, the incidence of hyperacute rejection was reduced[27-29]. This result indicated that simple GTKO in pigs is insufficient. Further genetic modification should be performed in the GTKO genetic background to construct more appropriate donors for xenotransplantation.

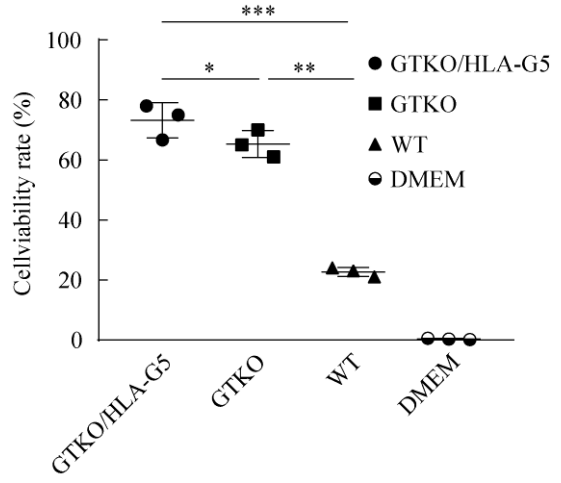

Figure 5 GTKO/HLA-G5 modification protects pig fibroblasts from human complement-mediated lysis. Porcine fibroblasts isolated from GTKO/HLA-G5, GTKO-only, and wild-type (WT) pigs were incubated in PBS with 10% normal human serum, and an MTT assay kit was used for detecting cell viability. G3, G9, and G14 are GTKO/HLA-G5 pigs. G1, G2, and G20 are GTKO-only pigs. The WT pigs are age-matched wild-type Bama mini-pig. Asterisks indicate a significant difference between two group (*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$).

HLA-G is a crucial immunosuppressive molecule and has been considered to contribute to human allograft acceptance. In other allogeneic situations, such as pregnancy, HLA-G expression has also been suggested to be related to feto-maternal compatibility. Thus, HLA-G can help shape the immune response towards tolerance in allogeneic and potentially xenogeneic transplantations[30]. A previous work reported the preparation of HLA-G1 transgenic swine endothelial cells (SECs)[31]. Co-culture of HLA-G1 transgenic SECs and macrophages led to significant suppression of macrophage-mediated SEC cytolysis. This work also demonstrated that transgenic HLA-G1 enhanced the expression of anti-inflammatory cytokines, and suppressed the expression of iNOS in co-cultured macrophages, revealing that transgenic HLA-G1 has widely inhibition in transplantation rejection[31]. HLA-E, similar to HLA-G, has been implicated in immune tolerance in transplantation and human reproduction. *In vitro* studies indicated that in HLA-E transgenic pigs, lymphocyte and endothelial cells showed anti-cytotoxic effects on NK cells; moreover, the expression of HLA-E on porcine endothelial cells has inhibitory effects on the secretion of IFN-γ[32]. The present work has important implications for the generation of HLA-G pigs to benefit xenotransplantation. Concerning the HLA-G isoform used for the transgenic pigs, HLA-G5 could be a better transgene candidate for its secreted form. Membrane-anchored HLA-G1 and secreted HLA-G5 proteins have equal immunosuppressive characteristics, and both represent the full extracellular length. Secreted HLA-G5 molecules could exert better immune regulating effect

through interaction with more immune cells.

In this study, we generated GTKO pigs expressing HLA-G5 via SCNT. PCR and Sanger sequencing indicated that all the 20 cloned piglets had homozygous mutations at the *GGTA1* gene loci. However, half of them were *HLA-G5* positive. Analysis of protein expression showed that Gal epitopes on the cell membrane of cells and tissues from GTKO/HLA-G5 piglets were completely absent, and HLA-G5 was successfully expressed in the cytoplasm of fibroblasts and different tissues from these gene-modified piglets. Functionally, MTT assay showed that the fibroblasts from the GTKO/HLA-G5 piglets displayed enhanced resistance to complement-mediated lysis compared with WT and GTKO-only pigs. All these data demonstrated that the GTKO/HLA-G5 pig was a candidate organ donor that was more beneficial for xenotransplantation than the GTKO-only pigs. The integration of the HLA-G into GTKO pigs would improve the success rates for the long-term survival of porcine xenografts, the use of which still needs further investigation through xenotransplantation trials in non-human primate recipients.

Acknowledgement

We thank the pathology laboratory for their assistance with pathological section and the animal cloning group in Guangzhou Institutes of Biomedicine and Health for their help in animal experiments.

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