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Generation and characterization of genetically modified pigs with GGTA1/β4GalNT2/CMAH knockout and human CD55/CD47 expression for xenotransfusion studies

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Pig red blood cells (pRBCs) represent a promising alternative to address the shortage in transfusion medicine. Nonetheless, a major obstacle to their clinical implementation is immunological rejection. In this study, we generated transgenic pigs expressing human CD47 (hCD47) and CD55 (hCD55) in α1,3-galactosyltransferase KO/β-1,4-N-acetyl-galactosaminyl transferase 2 KO/cytidine monophosphate-N-acetylneuraminic acid hydroxylase KO (TKO) pigs using CRISPR/Cas9 technology. Compared to wild-type pRBCs, TKO/*hCD47*/*hCD55* pRBCs exhibit significantly reduced human IgG/IgM antibody binding. Moreover, when transfused into Cynomolgus monkeys, TKO/*hCD47*/*hCD55* pRBCs remained detectable for 2 h post-transfusion, whereas wild-type pRBCs were eliminated within 20 min. This study demonstrates the potential of multi-gene edited pigs to provide immunologically compatible pRBCs.

Keywords Gene editing, Transgenic pigs, Xenotransfusion

Blood transfusion is a critical therapeutic intervention for saving patients' lives. However, the limited availability of blood resources poses a substantial barrier to its clinical implementation. According to the World Health Organization, approximately 112 million units of blood are donated globally each year, which is insufficient to meet patients' demands for blood products[1,2]. The escalating number of complex surgical procedures and emergency patients contributes to a constant increase in clinical transfusion, exacerbating the disparity between blood supply and demand. Furthermore, individuals with chronic blood disorders, such as sickle cell disease, require repetitive blood transfusions, which might trigger delayed hemolytic reactions or even hyper-hemolysis syndrome[3,4]. In areas with a high prevalence of blood-borne infectious diseases such as HIV and hepatitis, the economic burden of screening blood donors and the risk of infection for blood transfusions are significant concerns. To address these challenges, scientific research has long explored substitutes for human blood.

Recent advances in xenotransplantation involving genetically engineered pigs as organ donors have inspired the exploration of porcine blood products, such as pig red blood cells (pRBCs) and blood platelets, for clinical application[5–7]. pRBCs share numerous biochemical characteristics with human RBCs, including comparable cell size, count, and hemoglobin structure. As porcine RBCs and platelets lack cell nuclei, they do not express porcine leukocyte antigens or porcine endogenous retroviruses[8–10]. Rearing pigs in a specific pathogen-free environment allows pRBCs and platelet transfusions to avoid the risk of viral infections associated with human blood transfusions.

Despite the potential of pig blood as a novel source for clinical transfusion, several immune challenges must be overcome for its implementation. Wild-type (WT) pig blood cells express xeno-antigens that can be

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recognized by natural antibodies in human serum, leading to immune rejection[11]. The primary antigen targeted by natural antibodies in primates, known as αGal, can induce hyperacute rejection (HAR) in xenotransplantation procedures[12]. Pig blood cell surfaces also exhibit non-Gal antigens, including N-glycolylneuraminic acid (Neu5Gc) and Sd(a)[13,14]. The survival time of pRBCs transfused into non-human primates (NHPs) has not yet satisfied clinical requirements. WT pRBCs are eliminated within 15 min following transfusion into baboons. Eckermann et al.[15] reported that pRBCs lacking αGal antigens survived for less than 2 h in baboons. Transfusing small amounts of blood into baboons with depleted complement increased pRBC survival time to 24 h. Large-scale transfusion experiments in baboons revealed that pRBCs were predominantly phagocytosed by splenic macrophages[16]. These studies suggest that the rapid clearance of pRBCs in NHPs may be due to two factors: (1) the recipient's antibodies binding to antigens on pRBCs, subsequently activating the complement system and leading to pRBC lysis; (2) recipient macrophages phagocytosing pRBCs through antibody-dependent and/or -independent mechanisms.

A viable approach to addressing these challenges in the clinical application of pig blood is using genetically modified pigs. In our previous study, we successfully employed CRISPR/Cas9 technology to generate αGal, Neu5Gc, and Sd(a) triple knockout (TKO) pigs, which exhibited significantly decreased immunogenicity in their tissues and cells[17]. To extend the lifespan of TKO blood cells in NHPs, further genetic modifications are required. Xenograft transplantation research in NHPs has demonstrated that grafts expressing human complement regulatory proteins hCD55 (decay-accelerating factor) or hCD46 (membrane cofactor protein) exhibit a significant increase in resistance to complement-mediated damage[5,18–20]. The transfer of hCD47 molecules to pig cells can substantially inhibit the phagocytic activity of human macrophages against pig cells[21,22]. The survival period of pig hematopoietic progenitor cells in recipients was significantly extended by hCD47 in pig-to-human hematopoietic progenitor cell transplantation experiments in immunodeficient mouse models[23].

Therefore, we harnessed CRISPR/Cas9 technology to introduce humanized CD47 and CD55 genes under the control of erythroid-specific promoters into TKO pig fetal fibroblasts and subsequently constructed a TKO/*hCD47*/*hCD55* multi-gene modified pig model through somatic cell nuclear transfer. We assessed the gene expression and immunogenicity of TKO/h*CD47*/*hCD55* pig red blood cells and platelets and analyzed the survival of TKO/*hCD47*/*hCD55* pig red blood cells in non-human primates through xenotransfusion.

Results

Generation of TKO/*hCD47*/*hCD55* transgenic pigs using CRISPR/Cas9 technology

Our previous study generated *GGTA1*/*4GalNT2*/*CMAH* triple knockout (TKO) pigs and established TKO pig fetal fibroblast cells (PFFs) derived from 35-day-old TKO fetuses. To enhance pig-to-human transfusion compatibility, we synthesized a 978-bp human *CD47* coding sequence and an 1146-bp *CD55* encoding sequence, linked using a P2A sequence (see Fig. 1A). To facilitate the expression of *hCD47* and *hCD55* on the surface of pRBCs or platelets, we introduced an IHK chimeric promoter comprising an erythroid-specific enhancer from the human *ALAS2* intron 8, the human *HS40* core regulatory element, and the *ankyrin-1* promoter, or a human *CD100* promoter upstream of the *hCD47* and *hCD55* sequences, respectively. TKO PFFs were transfected with NHEJ-based *hCD47*/*hCD55* HITI vectors (Fig. 1A) and selected by puromycin for ten days. We isolated 96 resistant single-cell colonies and determined the *hCD47* and *hCD55* genotypes of each colony using PCR and Sanger sequencing with primers listed in Table 1. Of the 96 examined colonies, 30 were identified as *hCD47*/*hCD55* transgenic, yielding a positive rate of approximately 31.25%. We pooled these positive colonies as donor cells for the somatic cell nuclear transfer (SCNT) procedure and transferred approximately 200–300 reconstructed embryos to each surrogate pig. Ultimately, three successfully conceived recipients delivered four piglets. Subsequently, genomic DNA extracted from the ear tissue of the cloned piglets and the genotyping results indicated that they all bore mutations in the *GGTA1*, *β4GalNT2*, and *CMAH* target sites. Furthermore, the hCD100P-*hCD47*/*hCD55* vector was integrated into the p*Rosa26* locus, while the IHK-*hCD47*/*hCD55* sequence was randomly incorporated into the pig genome (Fig. 1B, S1).

TKO/*hCD47*/*hCD55* pigs express hCD47 and hCD55 on RBCs and Platelets

All the TKO/*hCD47*/*hCD55* piglets displayed no discernible differences in appearance compared to age-matched WT pigs. To verify the expression of hCD47 and hCD55, we isolated RBCs and platelets from the TKO/*hCD47*/*hCD55* piglets (n = 4) at one month of age. We then conducted the flow cytometry analysis to determine the frequency of $hCD47^+$ and $hCD55^+$ in each cloned piglet (Fig. 2). In the RBCs, the results showed 99.86% $hCD47^+$ and 97.85% $hCD55^+$ in human red blood cells, respectively, compared to 0.55% $hCD47^+$ and 0.10% $hCD55^+$ RBCs in a WT pig control. Although the ratios of $hCD47^+$ and $hCD55^+$ in erythrocytes of two cloned piglets were nearly negligible, a substantial expression of hCD47 and hCD55 was observed in the cloned piglets 2# and 3#, with $hCD47^+$ cells ranging from 1.59% to 2.0%, $hCD55^+$ cells ranging from 2.12% to 2.74%, and $hCD47^+$/ $hCD55^+$ cells ranging from 1.49% to 1.76% (Table 2). Compared to the WT control, all four genetically engineered piglets exhibited significantly increased $hCD55^+$ platelet fractions, ranging from 2.92% to 7.28% (Table 3). Notably, although the $CD47^+$ ratios of the cloned piglets 1#, 2#, and 3# were comparable to those of the WT pig platelets, a significantly higher $CD47^+$ proportion was detected in the cloned piglet 4# platelets. Overall, these results indicated detectable hCD47 and hCD55 expression on the RBCs and platelets of the cloned piglets, albeit with some variations.

TKO/*hCD47*/*hCD55* pRBCs significantly reduced human IgG and IgM antibody binding

Our previous studies demonstrated a significant reduction in the immunogenicity of porcine tissues upon deletion of three major glycan antigens, specifically αGal, Sd(a), and Neu5Gc[17,24]. To evaluate the immunogenicity of erythrocytes derived from TKO/*hCD47*/*hCD55* cloned piglets, RBCs were stained for human IgM and IgG binding and analyzed using flow cytometry. RBCs were incubated with 25% pooled human serum representing

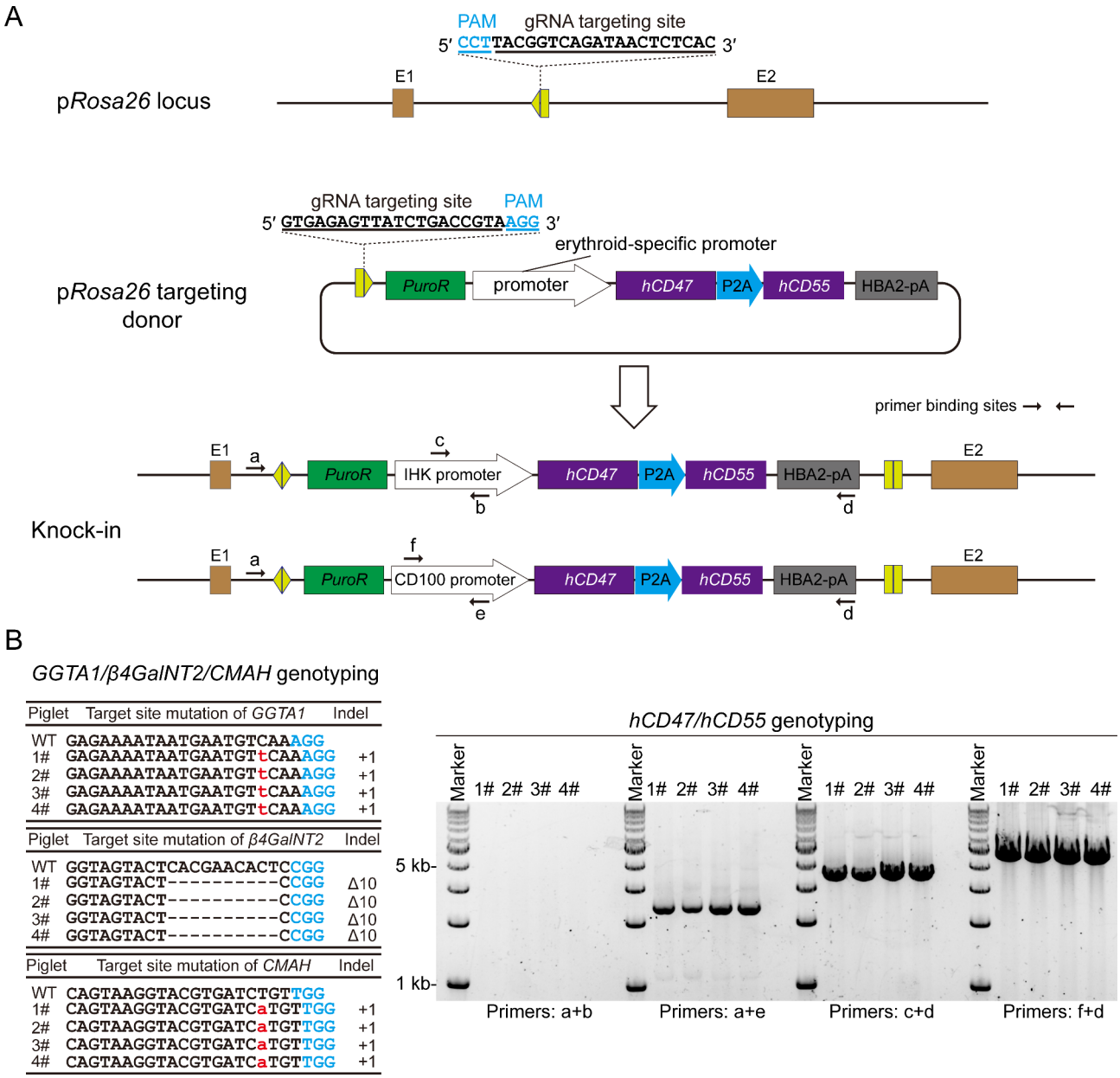

Fig. 1. Generation of the TKO/*hCD47*/*hCD55* multiple genetically modified piglets. (**A**) Design of CRISPR/Cas9-mediated NHEJ-directed homology-independent knock-in at the porcine *Rosa26* site. A schematic of the donor vector is shown, which contains the pig *Rosa26*-gRNA target site, an erythroid-specific promoter driving the *hCD47-P2A-hCD55* coding sequence, and a Puromycin resistance gene as a selection marker. The sgRNA sequences are shown in black and the PAM sequences are shown in blue. The gRNA-guided Cas9 concurrently cleaves the pig *Rosa26* target sequence and the donor plasmid to form double-strand breaks. NHEJ-mediated repair can directly ligate the DSB ends of the target and donor sequences. Two sets of primers (indicated by arrows) are used to amplify the donor and genome junction region and the donor fragment to confirm the targeted insertion of the IHK promoter-hCD47/hCD55 or CD100 promoter-hCD47/hCD55 donor vectors. (**B**) Genotyping results of *GGTA1*, *β4GalNT2*, and *CMAH* in the cloned pigs. The wild-type sequences are shown at the top, with sgRNA sequences in black and the PAM sequences in blue. '+' indicates insertion; 'Δ' indicates deletion (left). PCR results of *hCD47/hCD55* genotyping of the cloned pigs (right).

A, B, AB, and O blood types (n = 3 for each blood type) at 37 °C for 1 h to enable IgG binding, and 4 °C for one hour to facilitate IgM binding. This was followed by an incubation with anti-human IgG and IgM antibodies. RBCs obtained from WT pigs served as positive controls, while those isolated from the TKO pigs functioned as negative controls. The observed IgG-positive rates were 99.50% for WT RBCs, 0.93% for TKO RBCs, and 1.59% for TKO/*hCD47*/*hCD55* RBCs, respectively. The IgM-positive rates amounted to 99.30% for WT RBCs, 5.07% for TKO RBCs, and 12.23% for TKO/*hCD47*/*hCD55* RBCs, respectively. These findings suggested that,

Targets	Sequence (5′-3′)
GGTA1 F	CCTTAGTATCCTTCCCAACCCAGAC
GGTA1 R	GCTTTCTTTACGGTGTCAGTGAATCC
β4GalNT2 F	CCCAAGGATCCTGCTGCC
β4GalNT2 R	CGCCGTGTAAAGAAACCTCC
CMAH F	CTTGGAGGTGATTTGAGTTGGG
CMAH R	CATTTTCTTCGGAGTTGAGGGC
Primer a	TCATCGCCTCCATGTCAGTT
Primer b	TGGGGACCGCAGATTACAAG
Primer c	CTCCACTCCTGACCCTATCC
Primer d	GCTGGGACTCCCTGAGTAGA
Primer e	TCTGAGAATAGGGCTTCGGC
Primer f	GGCTCTTCCACAGTGAGGTC

Table 1. Primers used for *TKO/hCD47/hCD55* genotyping.

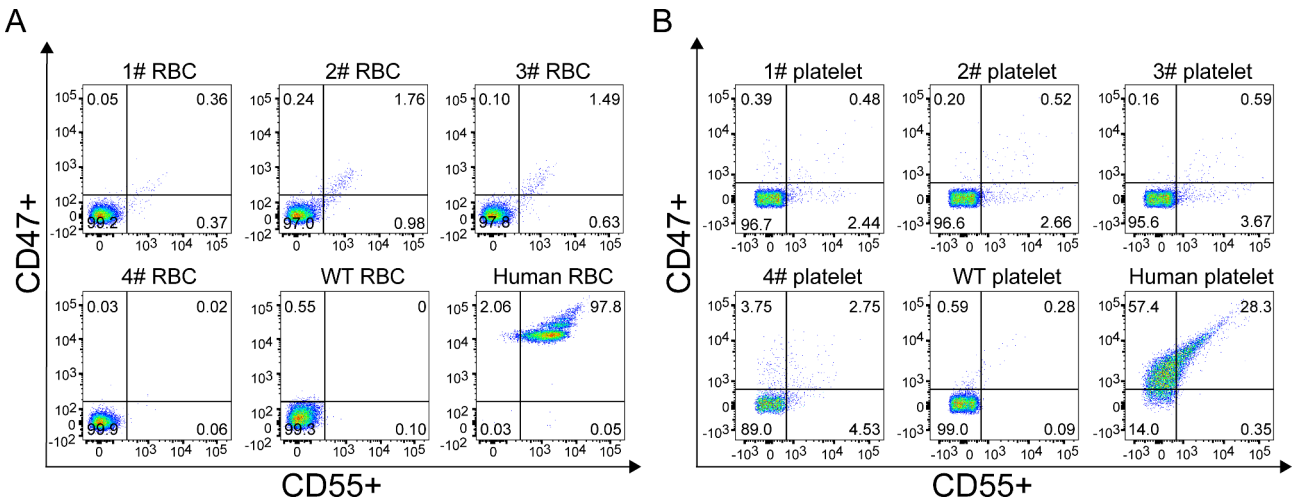

Fig. 2. Flow cytometry analysis of *hCD47* and *hCD55* expression on TKO/*hCD47*/*hCD55* pig RBCs and platelets. (**A**) Representative flow cytometric plots of RBCs with the quadrant numbers indicating the percentage of hCD47 and hCD55-positive cells. (**B**) Representative flow cytometric plots of platelets displaying the quadrant numbers indicate the percentage of hCD47 and hCD55-positive cells. The labels 1#, 2#, 3#, and 4# refer to individual TKO/*hCD47*/*hCD55* pigs.

	1# RBCs (%)	2# RBCs (%)	3# RBCs (%)	4# RBCs (%)	WT RBCs (%)	Human RBCs (%)
hCD47+	0.41	2.00	1.59	0.05	0.55	99.86
hCD55+	0.73	2.74	2.12	0.08	0.10	97.85
hCD47+hCD55+	0.36	1.76	1.49	0.02	0	97.8

Table 2. Statistics of hCD47 and hCD55-positive RBCs.

	1# platelets (%)	2# Platelets (%)	3# Platelets (%)	4# Platelets (%)	WT Platelets (%)	Human Platelets (%)
hCD47+	0.87	0.72	0.75	6.50	0.87	85.70
hCD55+	2.92	3.18	4.26	7.28	0.37	28.65
hCD47+hCD55+	0.48	0.52	0.59	2.75	0.28	28.30

Table 3. Statistics of hCD47 and hCD55-positive platelets.

compared to WT pigs, the binding of human IgG and IgM to RBCs from both TKO and TKO/*hCD47*/*hCD55* pigs was markedly reduced (Fig. 3).

Cynomolgus monkey IgG and IgM antibody binding assay

To perform porcine red blood cell transfusions into cynomolgus monkeys for preclinical investigations, we conducted immunological screening of recipient monkeys due to the variability in antibody levels against tissues from TKO pigs among non-human primates[25]. TKO/*hCD47*/*hCD55* erythrocytes were incubated with 25% monkey serum and subsequently assessed for binding levels to monkey IgG and IgM antibodies using flow cytometry. Erythrocytes from monkeys incubated with autologous serum served as negative controls. A total of nine monkeys were screened, with C2, C4, C7, and C8 exhibiting low levels of IgG and IgM binding to TKO/*hCD47*/*hCD55* RBCs (Fig. 4). Based on these findings, these four monkeys were chosen as recipients for xenotransfusion experiments.

TKO/*hCD47*/*hCD55* RBCs xenotransfusion in *Macaca fascicularis*

To monitor the infused RBCs in Cynomolgus monkeys, erythrocytes from WT pigs, TKO/*hCD47*/*hCD55* piglets, and Cynomolgus monkeys were labeled with carboxyfluorescein succinimidyl ester (CFSE). Subsequently, RBCs from pigs and Cynomolgus monkeys were transfused into four Cynomolgus monkey recipients (W.T.: n = 1, TKO/*hCD47*/*hCD55*: n = 3), with two autologous Cynomolgus monkey blood transfusions serving as controls. To evaluate erythrocyte survival time in recipients, CFSE-labeled erythrocytes in the blood were quantified by flow cytometry at 1, 2, 4, 6, and 24 h post-transfusion. The results demonstrated that CFSE-labeled monkey erythrocytes displayed negligible variation up to 24 h following autologous transfusion, whereas WT pig erythrocytes were predominantly eliminated within 20 min post-transfusion. Although a significant proportion of TKO/*hCD47*/*hCD55* erythrocytes were depleted 20 min post-transfusion, they were still detectable at 2 h post-transfusion (Fig. 5A). Furthermore, urine samples from Cynomolgus monkeys were analyzed after blood transfusion. Hematuria was identified within 15 min of WT pRBC infusion, while CFSE was detected in one WT pRBC-infused monkey and two TKO/*hCD47*/*hCD55* RBC-infused monkeys within 2 h of transfusion. The post-transfusion health status of the recipients was assessed by monitoring body temperature, blood biochemical and coagulation parameters. The monkey receiving WT pig erythrocytes exhibited shivers with a decreased body temperature of 37.8 °C (Fig. 5B). Additionally, this monkey developed hematuria, which was not observed in the other recipients. Prothrombin time (PT) in monkeys transfused with WT pig erythrocytes began to increase after one hour, thrombin time (TT), and lactate dehydrogenase (LDH) levels increased after 20 min, and gamma-glutamyl transferase (γ-GT) levels decreased after 20 min (Fig. 5C–F). These aberrations were not observed in recipient monkeys receiving the TKO/*hCD47*/*hCD55* RBC or autologous transfusions.

Discussion

With considerable progress achieved in pig-to-primate solid organ transplantation in recent years[26–29], the exploitation of genetically engineered pig blood to alleviate the shortage of clinical blood transfusions holds great promise. However, the efficacy of pig blood xenotransfusion has been hindered by the natural pre-existing antibodies in the recipient, complement-mediated lysis, and rapid clearance by macrophages. In this study, we generated multiple-gene modified piglets (TKO/*hCD47*/*hCD55*) by deleting αGal, Sda, and Neu5Gc, and inserting humanized hCD55 and hCD47 genes under erythrocyte-specific promoters. Compared to wild-type pigs, the xenotransfusion compatibility of the red blood cells and platelets from TKO/*hCD47*/*hCD55* piglets was enhanced, as demonstrated by reduced human IgM/IgG binding and prolonged survival time in non-human primates. These findings highlight the potential of these genetically modified pigs as a source of blood for xenotransfusion.

A critical measure of xenotransfusion success is the survival duration of pig RBCs in primates. In our study, wild-type pRBCs were rapidly cleared within 20 min post-transfusion in *Macaca fascicularis*, accompanied by adverse reactions such as hematuria, temperature loss, and altered blood physicochemical parameters. In contrast, a small percentage of TKO/*hCD47*/*hCD55* RBCs were still detectable 2 h after transfusion. This improvement, although modest, indicates reduced immunogenicity of the genetically modified pRBCs. Previous studies have reported varying survival times for genetically modified pig RBCs. For instance, αGal knocked out (GTKO) pRBCs survived for less than 2 h when transfused into baboons[15], while TKO RBCs survived over 100 h in capuchin monkeys[30]. The shorter survival time in our study may be attributed to the presence of Neu5GC in *Macaca fascicularis*, which likely enhances immune reactions against TKO cells. This is consistent with existing studies[25,31,32], indicating that Neu5Gc depletion in non-human primates, such as capuchin monkeys, may be more suitable for xenotransfusion studies.

A significant challenge encountered in this study was the insufficient expression levels of humanized hCD47 and hCD55 on pRBCs. We employed different promoters to drive the *hCD47/hCD55* expression cassette. However, the IHK chimeric promoter or hCD100-promoter could only achieve a slightly detectable expression level of hCD47/hCD55 in the peripheral blood cells. Prior studies have identified inadequate gene expression as a common challenge in transgenic cloned animals[23,33–35], influenced by promoter methylation and knock-in sites[36,37]. In our case, random insertion of the IHK-*hCD47/hCD55* donor may have reduced their expression, and the lack of recognition of IHK promoter and hCD100-promoter by the pig transcriptional machinery likely contributed to inefficient expression[38,39]. To address this, we removed the promoter of our vectors and introduced the modified vector into porcine kidney cells (PK-15) in front of CD47 exon 1, leveraging the endogenous porcine CD47 promoter. This approach significantly enhanced the expression of hCD55, suggesting that species-specific promoters are crucial for achieving adequate gene expression in transgenic animals (Fig. S2). Future studies should focus on refining transgene designs and exploring alternative promoters, such as the pig hemoglobin promoter and CD47 promoter, to achieve higher and more stable expression of humanized CD47 and CD55 genes.

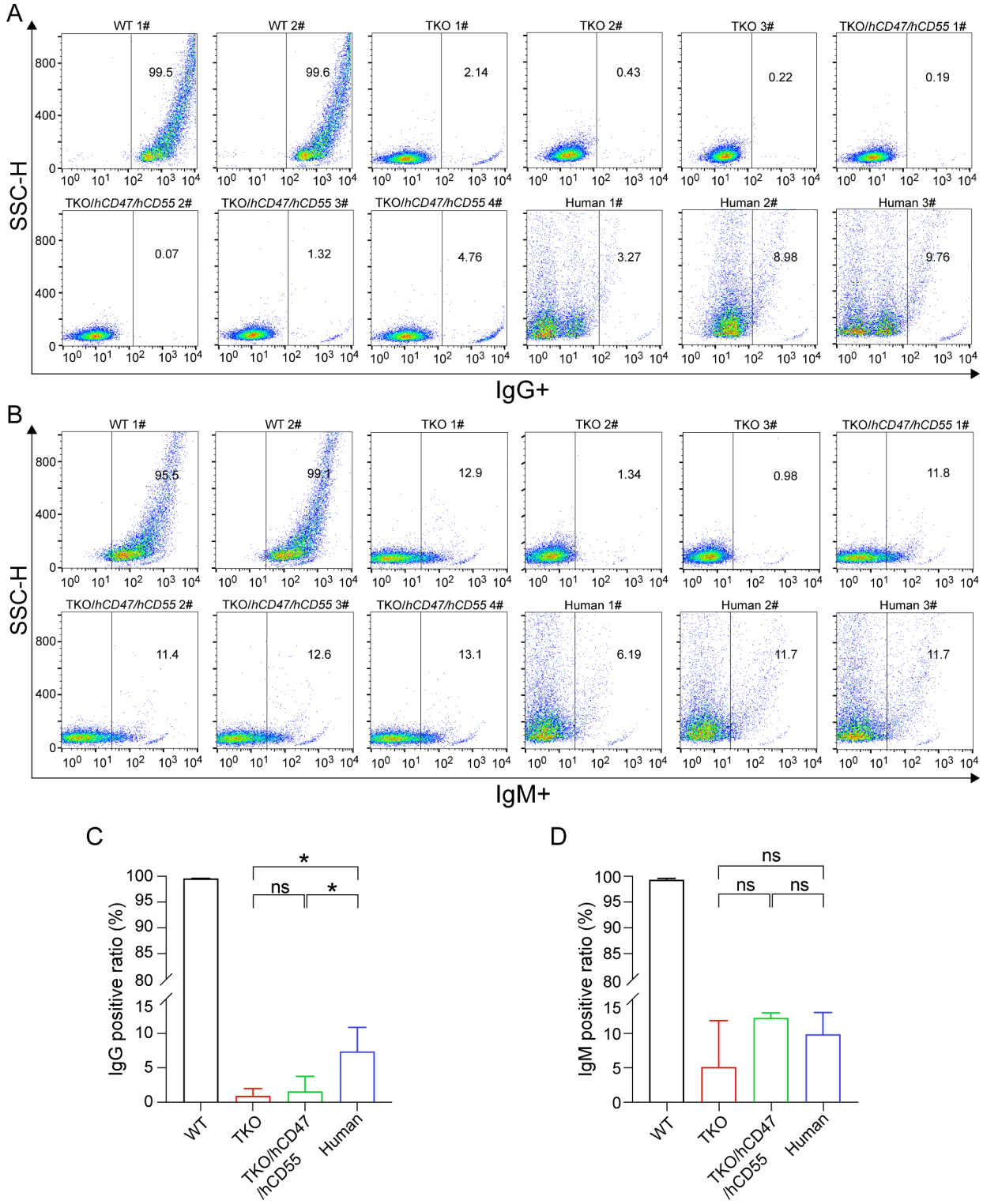

Fig. 3. Human IgG and IgM binding to pRBCs. (**A**) Flow cytometry analysis showing human IgG binding to RBCs from WT, TKO, TKO/*hCD47*/*hCD55* pigs, and human RBCs. (**B**) Flow cytometry analysis showing human IgM binding to RBCs from WT, TKO, TKO/*hCD47*/*hCD55* pigs, and human RBCs. (**C**) The statistical results show the proportion of RBCs bound by human IgG antibodies. One-way ANOVA was used to compare the groups, followed by Tukey's post hoc test. TKO vs TKO/*hCD47*/*hCD55* RBC ($P=0.03$), TKO vs human RBC ($P=0.03$), TKO/*hCD47*/*hCD55* vs human RBC ($P=0.04$). (**D**) The statistical results show the proportion of RBCs bound by human IgM antibodies. Kruskal–Wallis test was used to compare the groups, followed by Dunn's post hoc test. TKO vs TKO/*hCD47*/*hCD55* RBC ($P=0.48$), TKO vs human RBC ($P>0.99$), TKO/*hCD47*/*hCD55* vs human RBC ($P=0.79$). * $P<0.05$, ns: not significant.

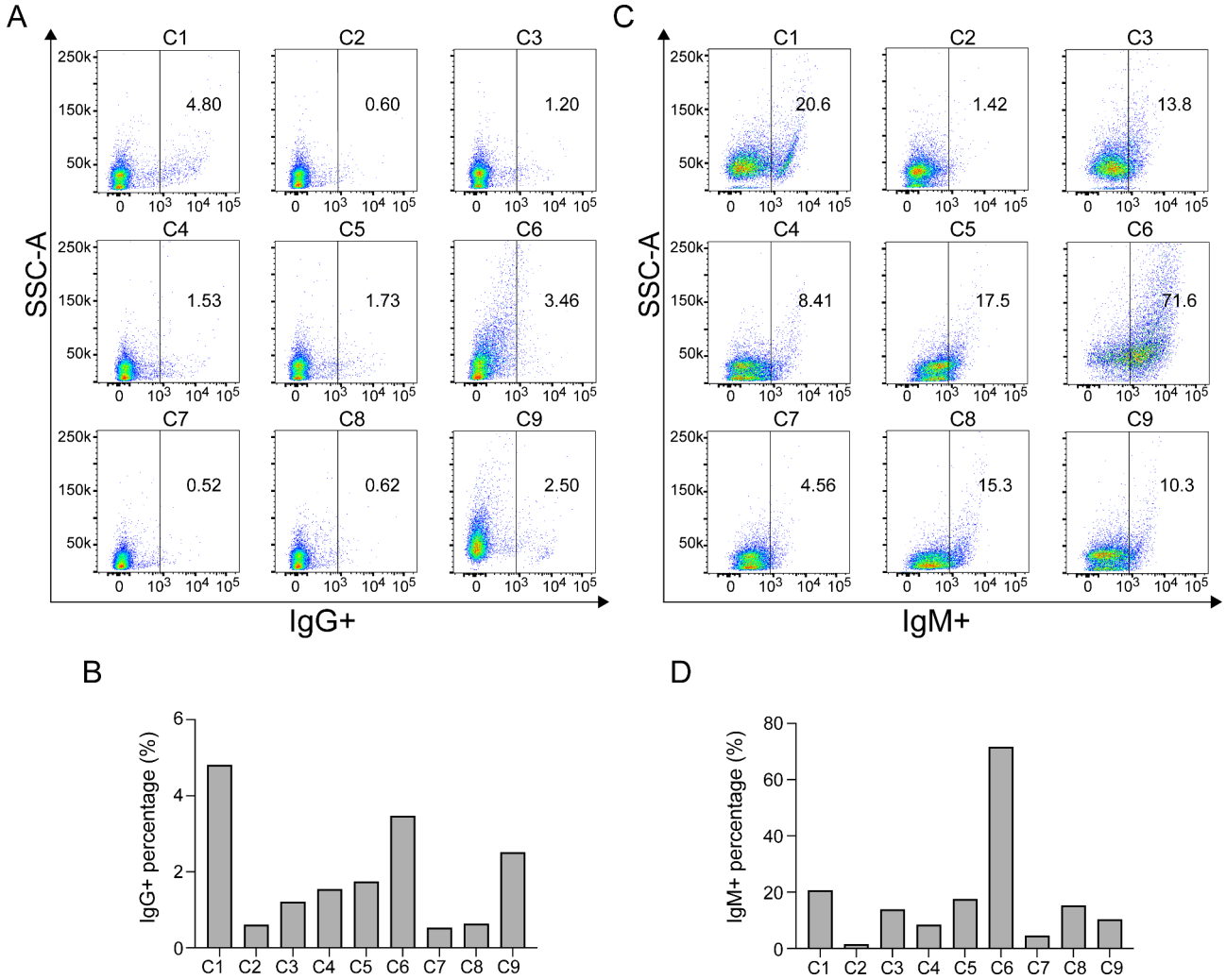

Fig. 4. Screening of Cynomolgus monkey serum IgM/IgG binding to TKO/*hCD47*/*hCD55* pRBCs. (**A**) Flow cytometry results show Cynomolgus IgG binding to pRBCs from different individuals. (**B**) Flow cytometry results show Cynomolgus IgM binding to pRBCs from nine individuals. (**C**) The statistical results show the proportion of pRBCs bound by Cynomolgus IgG antibodies across individuals. (**D**) The statistical results show the proportion of pRBCs bound by Cynomolgus IgM antibodies across individuals.

Several limitations must be acknowledged in our study. Firstly, the study design did not include TKO pig RBCs (without CD47 and CD55 expression) as a control in the in vivo trials due to resource constraints. Including this control in future studies would allow for a more precise determination of the impact of additional genetic modifications on pRBC survival. Secondly, although the 2-h survival time of TKO/*hCD47*/*hCD55* pRBCs represents a significant improvement, it falls short of the duration required for many clinical applications. However, in specific acute medical scenarios, such as emergency transfusions for severe hemorrhage or trauma, this extension in survival time may have clinical importance.

In summary, this study demonstrates the potential of multi-gene editing in pigs to produce pRBCs with enhanced survival and reduced immunogenicity in non-human primate models. By introducing humanized *CD47* and *CD55* into TKO pigs using CRISPR/Cas9, we developed hypoimmunogenic pRBCs that exhibited a prolonged two-hour post-transfusion circulation time, contrasting with the rapid elimination of WT pRBCs. Prior to clinical translation, substantial obstacles need to be addressed, including extending pRBC survival beyond 2 h and producing cost-effective large animal models on a larger scale. Despite these challenges, this study offers preliminary evidence that genetic engineering may facilitate the production of universal donor pRBCs. Further refinements will be necessary to address the limitations emphasized here and advance xenotransfusion toward a clinical reality through systematic optimization of multi-gene modifications, cloning techniques, and xenotransplantation protocols.

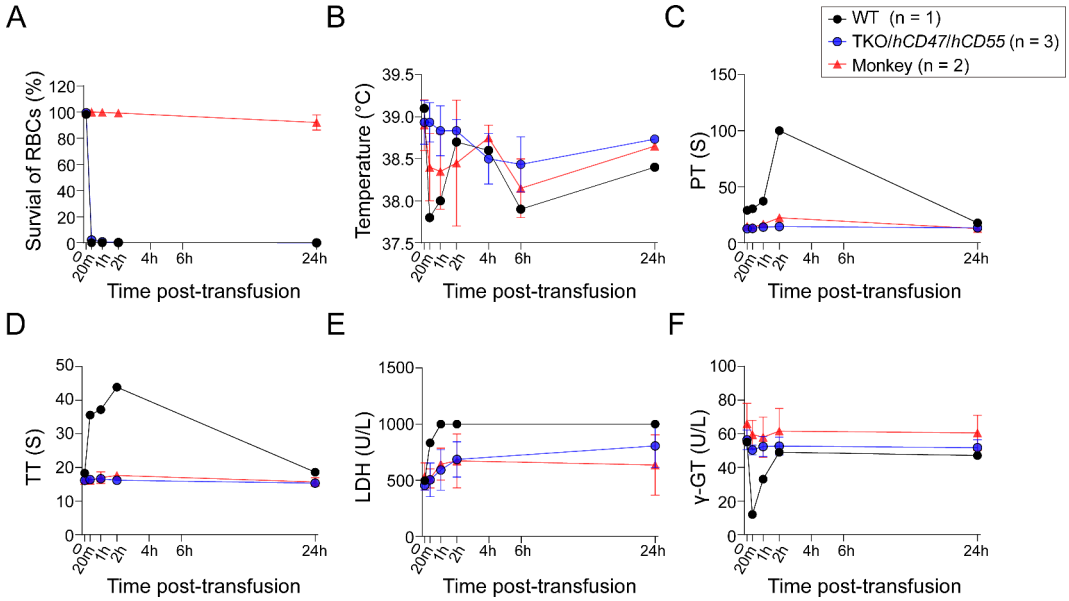

Fig. 5. Comparative analysis of xenotransfusion and allotransfusion outcomes. (**A**) Survival rates of RBCs following xenotransfusion and allotransfusion. (**B**) Recorded body temperature of Cynomolgus recipients post-xenotransfusion and allotransfusion procedures. (**C**) Prothrombin Time (PT) values of recipients after xenotransfusion and allotransfusion. (**D**) Thrombin Time (TT) measurements of recipients subsequent to xenotransfusion and allotransfusion. (**E**) Lactate Dehydrogenase (LDH) levels of recipients post-xenotransfusion and allotransfusion. (**F**) γ-GT concentrations of recipients following xenotransfusion and allotransfusion. For xenotransfusion, the recipient receiving WT pig RBCs (n = 1), and the recipients receiving TKO/hCD47/hCD55 pig RBCs (n = 3); for allotransfusion, the recipients receiving autologous Cynomolgus monkey RBCs (n = 2).

Methods and materials

Animals

All experiments were performed in accordance with relevant guidelines and regulations. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University and the Institutional Animal Care and Use Committee (IACUC) of Safe Pharmaceutical prior to the experiments. All methods are reported in accordance with ARRIVE guidelines. Porcine were purchased from the Charoen Pokphand Group (Taizhou, China) and housed in a large animal facility affiliated with Nanjing Medical University (Nanjing, China). Standard pig husbandry procedures were applied to all animals. The Cynomolgus monkeys were obtained from Safe Pharmaceutical (Shenzhen, China) and the blood transfusion assay were performed by Safe Pharmaceutical (Shenzhen, China).

Human blood samples

All experiments were performed in accordance with relevant guidelines and regulations. Experiments involving human blood were approved by the Ethics Committee of Jiangsu Province Blood Center. Whole blood was drawn from 12 healthy human volunteers (all ABO blood groups) after obtaining informed consent.

CRISPR/Cas9 targeting vector and hCD47/hCD55 donor plasmid construction

To target the porcine *Rosa26* gene, sgRNAs were designed using online tools (http://www.rgenome.net/cas-designer/). The DNA oligos of sgRNA were purchased from Sangon Biotech (Shanghai, China) (*Rosa26* sgRNA: 5′-CACCGGTGAGAGTTATCTGACCGTA-3′, 5′-AAACTACGGTCAGATAACTCTCAC-3′). The target of GGTA1, β4GalNT2, and CMAH was the same as described[17]. These oligos were mixed and incubated at 37 °C for 30 min, 95 °C for 5 min, and annealed at 25 °C at a rate of 5 °C/min. The annealed oligos were subcloned into pX330 (Addgene plasmid 423,230), which was digested by *Bbs*I (New England Biolabs, Beverly, USA). The donor plasmid of hCD47/hCD55 was purchased from Genscript (Nanjing, China).

PFF transfection, selection, somatic cell nuclear transfer (SCNT), and embryo transfer

To establish TKO/*hCD47/hCD55* cell lines, we transfected 2 μg of *IHK-hCD47/hCD55* donor, 2 μg of *hCD100P-hCD47/hCD55* donor, and 2 μg pX330 plasmid into 1×10^6 early passage of TKO PFFs (isolated from 35-day TKO embryos) by the Basic Fibroblast Nucleofection Kit (Amaxa Biosystems/Lonza, Cologne, Germany)[17]. We designed three pairs of primers for *TKO/hCD47/hCD55* genotyping. All primers are listed in Table 1. The oocytes were collected from six-month-old gilts and cultured for 42–44 h for maturation. The nucleus of mature oocytes was removed, and a single donor PFF was injected into the perivitelline space of the enucleated oocyte. After electrofusion, the reconstructed embryos were cultured at 38.5 °C for 24 h, and then 250–300 embryos

were transplanted to the surrogate sows. Single-donor PFF was injected into the enucleated oocyte, cultured at 38.5 °C for 24 h, and then transplanted to the surrogate sows.

Human, cynomolgus monkey, and porcine serum and RBC donors

Blood samples were obtained from healthy volunteers who had never undergone transfusion or transplant procedures. Blood was also collected from the veins of pigs. The blood type of the pigs was determined using PCR as described previously[7]. We specifically chose TKO/*hCD47*/*hCD55* pigs with the O blood type for our experiments. The whole blood samples were subjected to centrifugation at 500 g for 10 min at room temperature to separate the samples into serum and red blood cells (RBCs). The RBCs were washed thrice with PBS at 700 g for 5 min at 4 °C, and the serums were stored at -80 °C. The serums were dissolved at 4 °C prior to conducting assays[40].

Flow cytometry

RBCs (1×10^6 per sample) were stained at 4 °C for 1 h with Fluorescently or biotin-labeled antibodies to detect hCD47 and hCD55. The hCD47 was detected using Alexa Fluor® 647 Mouse Anti-Human CD47 (BD, Franklin Lakes, USA). To detect the hCD55, PE/Cyanine7 anti-human CD55 Antibody (Biolegend, San Diego, USA) was employed. To investigate the IgM/IgG binding to RBCs, the RBCs were incubated with 25% Cynomolgus monkey or human serums diluted with PBS at 37 °C for 30 min for IgG and at 4 °C for 30 min for IgM, respectively. Afterward, RBCs were stained for another hour at 4 °C using several specific antibodies: Goat anti-Human IgG Fc Highly Cross-Adsorbed Secondary Antibody, FITC (from Invitrogen, Grand Island, USA) for human IgG detection; FITC-Polyclonal Rabbit Anti-Monkey IgG (H + L) (from BioRab, Beijing, China) for *Macaca fascicularis* IgG detection; and Goat anti-Human IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, FITC (also from Invitrogen, Grand Island, USA) for both *Macaca fascicularis* and human IgM detection.

Staining RBCs of cynomolgus monkeys and pigs

Macaca fascicularis and pig RBCs were suspended in PBS and labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Grand Island, USA) for 15 min at 37 °C and washed twice in PBS at 4 °C[30].

Blood transfusion

Each *Macaca fascicularis* had 20 ml of their total blood substituted with pig RBCs labeled with CFSE or with their own RBCs similarly marked. Blood samples were collected at intervals of 1 h, 2 h, 4 h, 6 h, and 24 h following the transfusion. The RBCs and serum were separated to measure the fluorescence of the CFSE-marked RBCs and to examine serum biochemical indices[30].

Statistical analysis

The data from the quantitative experiments were first assessed for normality using the Shapiro–Wilk test. Variance equality was evaluated with the Brown-Forsythe test. For comparisons between two groups, a two-tailed Student's t-test was employed for normally distributed data, whereas the Mann–Whitney test was applied for non-normally distributed data. Statistical significance was determined by the analysis of variance (ANOVA) followed by post hoc multiple comparison tests (Dunnett's test or Tukey's test) or the Kruskal–Wallis test followed by Dunn's post hoc test for the analysis of the differences among three or more groups. Data are presented as means ± SD. A *P*-value of less than 0.05 was considered statistically significant.

Data availability

The data that support the findings of this study are available upon written request to the corresponding authors.

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Author contributions

H.Y. and Y.D. conceived and supervised the study; B.F., C.W., Y.Y., X.L., L.S., L.L., and Y.W. performed the experiments; B.F. and H.Y. wrote the manuscript; All the authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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