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RNA interference of GGTA1 physiological and immune functions in immortalized porcine aortic endothelial cells

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ARTICLE INFO

Article history:
Received 28 January 2013
Received in revised form
27 April 2013
Accepted 22 May 2013
Available online 14 June 2013

Keywords:
Xenotransplantation
α-1,3-Galactosyltransferase
RNA interference
Nature antibody
Complement

ABSTRACT

Background: Pig organs are commonly used in xenotransplantation, and α-1,3-galactose has been shown to be the main cause of hyperacute rejection. The development of transgenic pigs that lack α-1,3-galactosyltransferase (GGTA1) has overcome this problem to a certain extent, but transgenic pigs are difficult to maintain, making their usefulness in basic research limited. For this reason, we propose to establish a cell model to study hyperacute rejection.
Methods: Immortalized primary porcine aortic endothelial cells were transfected with a short hairpin RNA targeted to GGTA1. Cell proliferation, apoptosis, complement C3 activation, and the binding of human immunoglobulins and components of the complement system, including IgM, IgG, C3, and C5b-9, were examined.
Results: After RNA interference, GGTA1 was found to be reduced at both the transcript and protein level as assessed by quantitative polymerase chain reaction and flow cytometry, respectively. When cultured in the presence of human serum, the proliferation rate of the transfected cells was higher than that of untransfected cells, and the apoptosis rate was lower. Additionally, activation of C3 and the binding of human immunoglobulins IgM and IgG and complement component C3 and C5b-9 to the transfected cells were lower than in the immortalized group but higher than in untransfected cells.
Conclusions: RNA interference of GGTA1 in cultured porcine endothelial cells reduces the reaction of immunoglobulin and complement system with the cells. Therefore, this *in vitro* cell model could be useful for further study of xenotransplantation.

1. Introduction

Xenotransplantation—the replacement of human organs with animal organs—is a relatively common approach to address organ deficiency or failure [1]. However, xenograft rejection, molecular incompatibility, and interspecific infection restrict the clinical use of xenotransplantation. Among these issues, xenograft rejection is of highest importance [2–6]. Xenograft rejection includes hyperacute rejection, acute rejection, and chronic rejection [4]. Hyperacute rejection results from the interactions of natural antibodies in human serum (HS) with α-1,3-galactose (α-1,3Gal) on porcine aortic endothelial cells

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http://dx.doi.org/10.1016/j.jss.2013.05.080

(PAECs) [7]. In 2003, Phelps *et al.* [8] established the first α-1,3-galactosyltransferase (GGTA1)-knockout pigs to overcome hyperacute rejection. In recent years, transplantation using organs from multi-transgenic pigs has yielded remarkably prolonged graft survival [9,10]. However, because transgenic pigs are limited, expensive, and difficult to maintain, they are not appropriate for basic immune studies or immune therapy. As an alternate approach, cell models that could simulate xenograft rejection would allow observation of pathologic changes, identification of the molecules involved, and confirmation of proposed molecular mechanisms. We therefore used RNA interference (RNAi) to establish a GGTA1-deficient PAEC line to examine immune and physiological functions in the presence of HS. The goal was to establish a cell model to aid in reducing molecular reaction and allow further study of this process.

2. Materials and methods

2.1. Extraction of PAECs

PAECs were obtained following the methods described by Seebach *et al.*, as follows [11]. Approximately 30-d-old pigs (~3 kg each) were sacrificed by phlebotomy, and approximately 10 cm of their aortic tissue was obtained under sterile conditions. Specimens were washed with phosphate-buffered saline (PBS) at 4°C, and small blood vessels were ligated with suture line. Specimens were then filled with harvesting solution, which was Dulbecco's modified Eagle medium (DMEM) supplemented with 0.2% collagenase type I (Gibco, Grand Island, NY), 100 U/mL penicillin, and 100 mg/mL streptomycin and incubated at 37°C under sterile conditions for 40 min. Cells were collected by flushing the aortic lumen with harvesting solution, then they were washed and plated in complete medium (CM), which was DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, 100 mg/mL streptomycin, 100 mg/mL L-glutamine, 10 mM HEPES, and 50 μg/mL vascular endothelial cell growth factor (Sigma, St. Louis, MO). Cells were grown on gelatin (Sigma)-coated culture flasks and detached for passaging with 0.25% trypsin when necessary.

2.2. Uptake of acetylated low-density lipoproteins

The uptake of acetylated low-density lipoproteins (Ac-LDL) was assessed to identify the endothelial cells by incubating PAECs with 5 mg/mL 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine-labeled Ac-LDL (DiI-Ac-LDL) (Molecular Probes, Grand Island, NY) in medium without serum for 12 h at 37°C. After removing the medium, cells were washed three times in PBS and observed through an inverted fluorescence microscope.

2.3. Establishment of cell line expressing simian virus 40 large T antigens and GGTA1-shRNA

PAEC lines (passages 2–5) were transformed by lentivirus transfection using a four-plasmid lentiviral system (pLenti-CMV SV40LT, PLP1, PLP2, VSV-G) kindly provided by Dr Nie Yong-zhan of the Xijing Hospital of Digestive Diseases. Cells transfected with simian virus 40 large T antigens (SV40LT) were then transfected with a short hairpin RNAs (shRNA) targeted to GGTA1. Four different shRNA were designed; the sequences are shown in Table 1.

A random mismatched sequence, which is called GGTA-RNAi-NC, was chosen as mock-transfected; its sequence is also shown in Table 1. Each individual shRNA was ligated into the GV112 vector (hU6-MCS-CMV-puromycin) and encapsulated within lentivirus. The synthesis, ligation, and encapsulation of shRNAs were performed by GeneChem Co Ltd (Shanghai, China).

2.4. Transfection of cells

Cells were cultured at 37°C in 24-well plates with 2×10^4 cells in each well. Medium was removed at 24 h, and 1 mL DMEM was added together with 2×10^5 lentivirus. Viral medium was replaced by CM 10 h later. Cells were then transferred to 6-well plates. After 3 d, 0.2 mg/mL G418 or 2 ng/mL puromycin was added. Then cells were kept at 37°C for another 3 d. Cells were observed until no cells could be seen in the control group. The remaining cells were cultured for further study.

2.5. Real-time polymerase chain reaction of SV40LT and GGTA1

Total RNA was extracted from PAECs using Trizol reagent (Invitrogen, Grand Island, NY). Complementary DNA was synthesized from 1 μg total RNA in a 20-μL reaction using PrimeScript RT reagent kit (Takara Bio Inc). Real-time polymerase chain reaction (PCR) was performed on an IQ5 (BioRad, Hercules, CA), and the data were analyzed on the IQ5 Detection System (Version 2.0). The specificity of the amplified products was checked by melting analyses. SYBR Green

Table 1 – Sequences for shRNA.

GGTA1-RNAi#1	Sense:	ccggaa(GGTTCTTTGTTCTGGATAT)CTCGAG(ATATCCAGAACAAAGAACC)TTTTTTTg
	Antisense:	aattcaaaaaaa(GGTTCTTTGTTCTGGATAT)CTCGAG(ATATCCAGAACAAAGAACC)TT
GGTA1-RNAi#2	Sense:	ccggga(GGTGGCAAGACATCAGCAT)CTCGAG(ATGCTGATGTCTTGCCACC)TCTTTTTg
	Antisense:	aattcaaaaaga(GGTGGCAAGACATCAGCAT)CTCGAG(ATGCTGATGTCTTGCCACC)TC
GGTA1-RNAi#3	Sense:	ccggca(GCCCAGAAGGTTCTTTGTT)CTCGAG(AACAAAGAACCTTCTGGGC)TGTTTTTg
	Antisense:	aattcaaaaaca(GCCCAGAAGGTTCTTTGTT)CTCGAG(AACAAAGAACCTTCTGGGC)TG
GGTA1-RNAi#4	Sense:	ccggaa(GGAAGAGTGGTTCTGTCAA)CTCGAG(TTGACAGAACCACTCTTCC)TTTTTTTg
	Antisense:	aattcaaaaaaa(GGAAGAGTGGTTCTGTCAA)CTCGAG(TTGACAGAACCACTCTTCC)TT
GGTA-RNAi-NC	Sense:	CCGG(TTCTCCGAACGTGTCACGT)TTCAAGAGA(ACGTGACACGTTCGGAGAA)TTTTTG
	Antisense:	AATTCAAAAA(TTCTCCGAACGTGTCACGT)AAGTTCTCT(ACGTGACACGTTCGGAGAA)

Premix (Takara Bio, Shiga, Japan) was used for all experiments. Experiments were conducted in triplicate, and the relative quantification model was applied to calculate target gene expression relative to that of GAPDH internal control. Primers for real-time PCR are shown in Table 2. The PCR parameters for 20-μL reactions included denaturation at 95°C for 3 min, then 40 cycles at 95°C for 10 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. Fluorescence data were collected at each cycle after the elongation step.

2.6. Cell proliferation assay

Cells were collected and cultured in 96-well plates with CM. After cells adhered to the plates, CM was replaced with medium containing 5% or 10% commercial HS (Zhongkechenyu Ltd, Beijing, China) or FBS. Cells were incubated at 37°C for 24 h, and cell proliferation was assessed by MTT assay every 4 h to derive cell growth curves.

2.7. Flow cytometry to detect α-1,3-Gal and apoptosis

The relative abundance of α-1,3-Gal in cells and the extent of apoptosis were examined by flow cytometry. Approximately 5×10^5 cells from each treatment were collected by centrifugation and resuspended in PBS with 0.1% bovine serum albumin. Fluorescein isothiocyanate (FITC)-conjugated Griffonia simplicifolia isolectin B4 (GS-IB4, 2 μg/mL; Sigma) (a carbohydrate-binding lectin) was added to each sample, after which samples were incubated for 45 min at 4°C, then analyzed by flow cytometry (Becton Dickinson, San Jose, CA). Cells were harvested before and 24 h after exposure to serum. The Phycoerythrin and FITC Double-labeled Annexen V kit (Invitrogen) was used for detection of apoptosis. The average fluorescence intensity of each sample was recorded.

2.8. Immunofluorescence and immunocytochemistry

Immortalized cells and GGTA1-interfered cells were cultured on 24-well plates with 5% or 10% HS or 10% FBS for 24 h. Then, cells were washed three times in PBS, fixed in 4% paraformaldehyde, permeabilized by 0.1% (vol/vol) Triton-100, and sealed by 0.3% hydrogen peroxide and 10% goat serum. Cells were then incubated with rabbit anti-pig von Willebrand factor (vWF) (PL Laboratories, British Columbia, Canada) (1:100), goat anti-human complement C3 (Biosis, Beijing, China) (1:100), or rabbit anti-human complement C5b-9 (Abcam) (1:150) at 4°C overnight. FITC-labeled goat anti-rabbit secondary antibody together with a 1:1000 dilution of 4′,6-diamidino-2-phenylindole, or horseradish peroxidase (HRP)-labeled mouse anti-goat secondary antibody (1:200), HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas, Texas) (1:150), or Texas Red–labeled IgG (Abcam) or Alexa Fluor 488–labeled IgM (Abcam) were added to the sample. The DAB coloration kit (Zhongshanjinqiao Ltd Co) was used where necessary. Immortalized cells and mismatched shRNA-transfected cells were used as controls. Ratios of immunocytochemistry- or immunofluorescence-positive cells were used for comparison.

Table 2 – Sequences for all primers in real-time PCR.

SV40LT	Sense:	5′CAAAGTGTGGTATGGCTGAT3′
	Antisense:	5′GGCTACTGGGAACTGGA3′
α-1,3 GT	Sense:	5′GAAGAGGTGGCAAGACATCAG3′
	Antisense:	5′CTCGTAGGTGAACTCGTCAGG3′
GAPDH	Sense:	5′ATGGTGAAGGTCGGAGTGAA3′
	Antisense:	5′TGGGTGGAATCATACTGGAAC3′

2.9. Western blotting

Reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using 8% sodium dodecyl sulfate–polyacrylamide gels. Supernatant from immortalized cells, mock-transfected cells, or transfected cells incubated with 5% HS were tested. All samples were reduced by mercaptoethanol (Sigma) and rainbow Molecular Weight Protein Markers (Sigma) were included. After the sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the separated proteins were blotted onto nitrocellulose membrane (Millipore, Billerica, MA). The membranes were then blocked with 5% nonfat dry milk at room temperature for 1 h, and the membranes were incubated overnight at 4°C with the anti-C3/C3a antibody (1:150, ab11874; Abcam), which targeted the C3a on the α-chain of the C3; anti-C3 antibody (1:200, ab17455; Abcam), which targeted the β-chain of C3; and β-actin (1:2000; Sigma). After washing three times in TBST, the membranes were incubated with HRP-conjugated anti-rabbit and anti-mouse IgG secondary antibodies (1:3000; Sigma) for 1 h at room temperature. Immunoblots were detected with an enhanced chemiluminescence system (BioRad) according to the manufacturers' protocol.

2.10. Enzyme-linked immunosorbent assay

Assays of natural antibody are similar to those described by Platt *et al.* [12]. First, 1×10^3 immortalized cells, mock-transfected cells, and GTKO cells, respectively, were seeded in each well of the 96-well plates and incubated in 5% CO_2, 37°C for 24 h. Then supernatants were removed and cells were fixed by 200 μL cold 0.01% glutaraldehyde for 5 min at 4°C. Then nonspecific proteins were blocked with 1% bovine serum albumin at 25°C for 1 h. Serial dilutions of HS were added to each and incubated for 1 h at room temperature. Biotinylated goat anti-human IgM or IgG secondary antibodies (Abcam; diluted 1:3000) and streptavidin alkaline phosphatase were added and incubated at room temperature for 1 h. Finally, substrate solution (p-nitrophenyl phosphate in 0.1 M dietanolamin buffer, pH 9.6) was added and developed in the dark at 37°C for 10–20 min. Optical density was read at 405 nm using Epoach Reader (Biotek, Winooski, VT).

2.11. Statistics

The statistical tests were carried out using PASW statistics 18 (IBM Co, Armonk, NY). All results are presented as mean ± standard deviation. Significant differences between two groups were determined by the Student *t*-test and the LSD *t*-test. Significant differences between three groups were determined by one-way analysis of variance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Establishment and characterization of immortalized PAEC lines

Primary endothelial cells were obtained from the porcine aortic vessels and endothelial cells were immortalized by transfection of SV40LT. The expression of SV40LT was measured by real-time PCR. Immortalized cells expressed significantly more SV40LT than primary cells ($P < 0.05$; Fig. 1-I). Primary cells adhered to the culture flask within 24 h after derivation and displayed typical cobblestone morphology with spindle-shaped or spherical cells showing aggregative growth and fewer apophyses on the cell surfaces than immortalized cells (Fig. 1-IIA and B).

Under the transmission microscope, the endothelial cells showed similar ultrastructure before and after SV40LT transfection. Typical Weibel-Palade bodies could be seen in both cells, but a higher proportion of lipid droplets was observed in the immortalized cells (Fig. 1-IIC and D).

Immunofluorescence to detect the presence of vWF and the uptake of DiI-Ac-LDL were carried out before and after immortalization and revealed that the immortalization process did not change the general character of the PAECs. As shown in Figure 1-IIE, F, G, and H, both cell lines could take up DiI-Ac-LDL and express vWF in the cytoplasm.

3.2. Downregulation of GGTA1 by shRNA

We established four different shRNAs targeting GGTA1 and a mock group targeting random mismatched sequence and connected them to lentivirus, respectively. We designed 4 different shRNA (as shown in Table 1). Different shRNA targeted differently part of GGTA1 and the interference efficiency may be different. Immortalized cells were transfected with lentivirus and GGTA1 expression was detected by real-time PCR. As shown in Figure 2A, relative to GAPDH, the control had an expression rate of 1.088 ± 0.148 whereas the mock-transfected cells had an expression rate of 1.072 ± 0.129. Transfection with the four shRNA-containing sequences from the GGTA1 gene (GGTA1 #1–4) resulted in relative expression rates of 0.396 ± 0.067, 0.337 ± 0.074, 0.202 ± 0.055, and 0.432 ± 0.088, respectively. There were no significant differences between the untransfected and mock-transfected controls ($P = 0.800$), but there were significant differences between the controls and each of the four GGTA1 transfection groups ($P < 0.05$). When comparing among the transfected groups, the expression of GGTA1 in the GGTA1 #3–transfected cells was found to be significantly lower than in cells transfected with GGTA1 #1 or GGTA1 #4 ($P < 0.001$) and slightly lower than in cells transfected with GGTA1 #2 ($P = 0.051$). We therefore chose the cells transfected with GGTA1 #3 for further study.

3.3. Downregulation of α-1,3Gal after transfection

The relative abundance of α-1,3Gal in the control and GGTA1 #3–transfected cell lines was measured *via* flow cytometry using FITC-labeled GS-IB4. The fluorescence intensity of the transfected group was significantly lower than that of the immortalized cells and mock-transfected controls ($t = 35.78$, $P < 0.001$; $t = 36.52$, $P < 0.001$) (Fig. 2B). There was no significant difference in α-1,3Gal between the immortalized cells and mock-transfected groups ($t = 0.802$, $P = 0.404$).

3.4. Inhibition of cell proliferation by HS

Then cells were cultured with 5% or 10% HS, and proliferation was assessed by the MTT assay. In the 5% HS cultures, the absorbance of both the immortalized cells and mock-transfected controls was lower than the GGTA1-transfected group (Fig. 3). The difference between the two controls was not statistically significant ($P = 0.185$), whereas differences between the GGTA1-transfected group and the controls were significant (both $P < 0.001$) at 16 h after exposure to HS. In the 10% HS cultures, the growth curves of the immortalized cells and mock-transfected controls were similar but sharper,

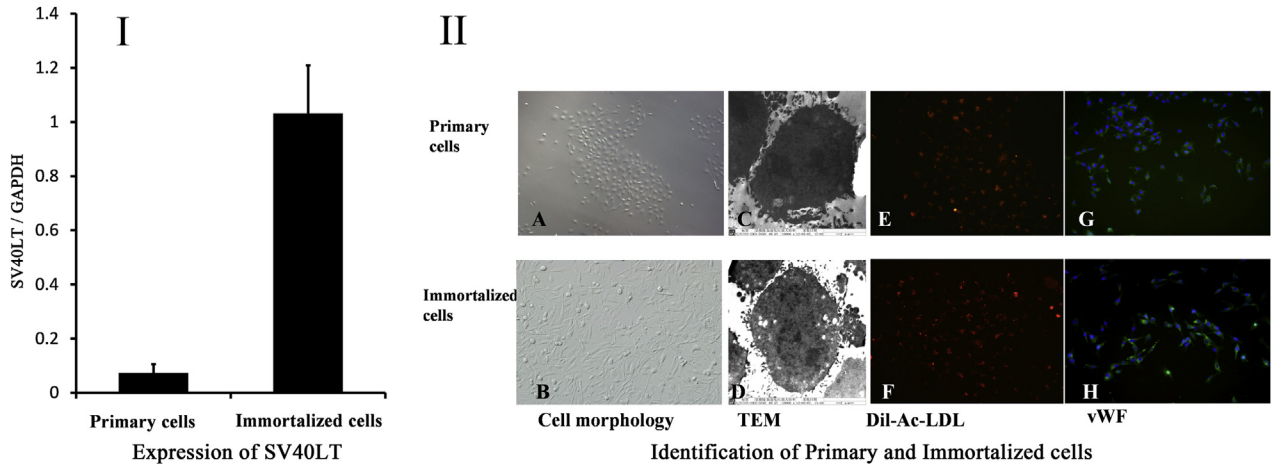

Fig. 1 – The expression of SV40LT in primary cells and immortalized cells (I) and identification of primary and immortalized cells, including: phase contrast microscopy (IIA, IIB); transmission electron microscopy (IIC, IID); uptake of DiI-Ac-LDL (IIE, IIF); and expression of vWF (IIG, IIH). (Color version of figure is available online.)

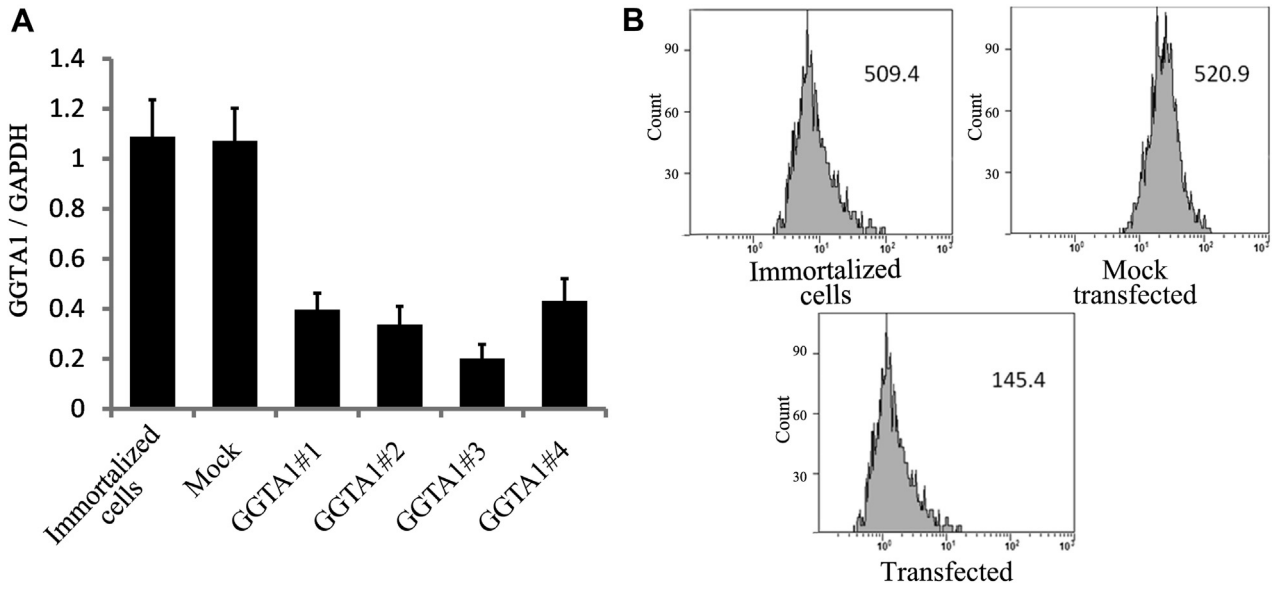

Fig. 2 – The expression of GGTA1 and α-1,3Gal after transfection of shRNA. Analyzed are (A) expression of GGTA1 in five different kinds of transfected cells and control cells; (B) expression of α-1,3Gal in control, mock-transfected control, and transfected group.

whereas the proliferation of the GGTA1-transfected group decreased slightly, rather than increasing as with the 5% HS culture. After 16 h in 10% HS, there was no significant difference between the two controls ($P = 0.224$), but differences between the GGTA1-transfected group and the controls were significant (both $P < 0.001$).

3.5. *Reduced apoptosis after culture with HS*

After culture with 5% HS for 16 h, apoptosis rates varied in the different groups (Fig. 4). Differences between the GGTA1-transfected group and the immortalized cells and mock-transfected controls were significant ($P = 0.004$, $P = 0.001$). Similar results were observed after culture with 10% HS for 16 h, but the extent of apoptosis in all groups was higher in 10% HS. Apoptosis in the GGTA1-transfected group was significantly lower than in the controls (both $P < 0.001$), whereas there was no significant difference between the two controls ($P = 0.575$). These results suggested that interference with GGTA1 provides a protective function in cells cultured in the presence of HS. On the other hand, apoptosis in the GGTA1-transfected group was much lower in 10% FBS than in 10% HS ($P = 0.047$), confirming previous observations.

3.6. *Binding of human immunoglobulin to PAECs reduced after RNAi targeting GGTA1*

The binding of human immunoglobulins (IgG and IgM) to cells before and after transfection in the presence of HS was examined by immunofluorescence and enzyme-linked immunosorbent assay (ELISA). As shown in Figure 5, the immortalized cells and mock-transfected controls reacted strongly with antibodies to human IgG and IgM, but this binding was greatly reduced in the GGTA1-transfected group. In immunofluorescence, the percentage of cells binding IgM in the GGTA1-transfected group was 34.7 ± 4.68, compared with 87.88 ± 5.37 ($P < 0.001$) in the immortalized cells and 81.94 ± 5.97 ($P < 0.001$) in the mock-transfected cells (Fig. 5A). The percentage of cell number binding IgG in the GGTA1-transfected cells was 62.26 ± 7.85, compared with 88.36 ± 5.70 ($P < 0.001$) in the immortalized cells and 92.22 ± 3.92 ($P < 0.001$) in the mock-transfected cells (Fig. 5B).

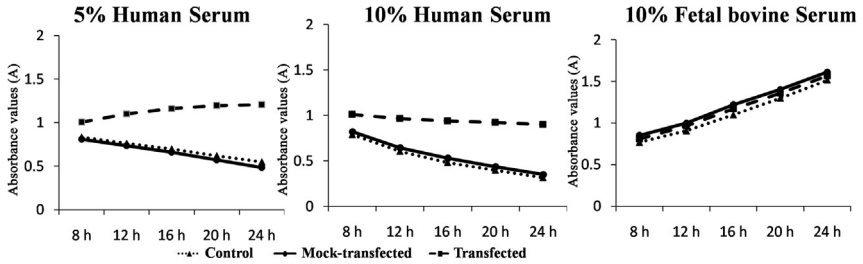

Fig. 3 – Proliferation of cells cultured in 5% or 10% HS and 10% FBS. Statistically significant differences among three cell lines were found both in 5% and 10% HS (all $P < 0.001$).

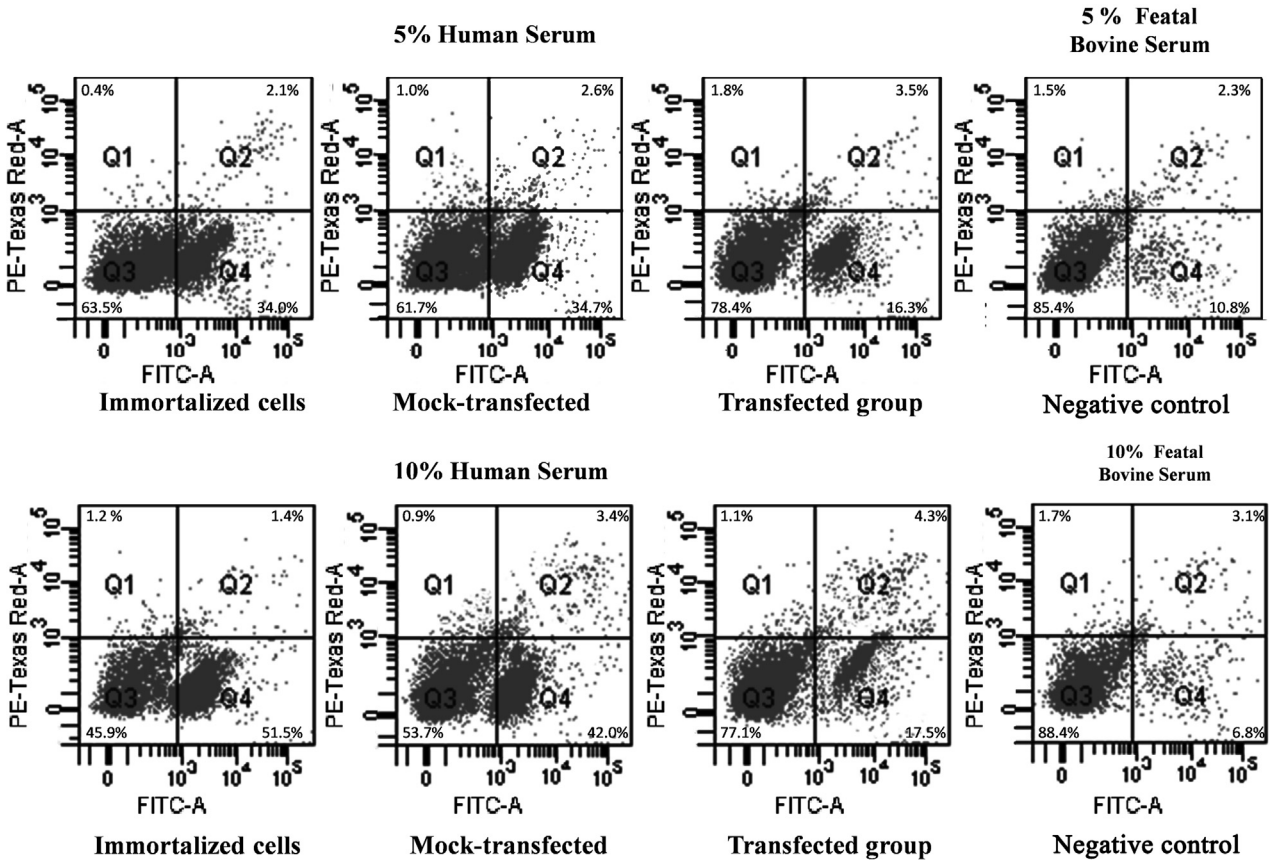

Fig. 4 – Apoptosis of different cells in 5% and 10% HS or in 10% FBS as negative control. Cells in FBS had the lowest apoptosis rate. Transfected group is the second-lowest, whereas mock-transfected and immortalized cells had the higher rate.

In ELISA test, reactivity of PAECs with human natural antibodies varied from cell to cell. At the concentration of 1:8, immortalized cells (IgM 1.156 ± 0.081; IgG, 1.531 ± 0.097) had similar OD (A405) to the mock-transfected cells (IgM, 1.017 ± 0.108, $P = 0.101$; IgG, 1.437 ± 0.114, $P = 0.205$), which were both higher than that of transfected cells (IgM, 0.547 ± 0.0.118; IgG, 0.831 ± 0.072) (both $P < 0.001$). But A405 of transfected cells were still higher than those of negative control groups, which demonstrated that there were still some human natural antibodies binding to the cells.

3.7. *Reactivity of human complement proteins to PAECs reduced after RNAi targeting GGTA1*

We next examined the relative binding and activation of human complement system proteins to the different groups by immunocytochemistry and Western blot. As shown in Figure 6A, the percentage of cells binding C3 in the GGTA1-transfected group was 38.62 ± 8.20, compared with 81.62 ± 7.65 ($P < 0.001$) in the immortalized cells and 76.84 ± 6.56 ($P < 0.001$) in the mock-transfected cells. The distribution of C5b-9 in the different groups was similar to C3. The percentage of cells binding C5b-9 in the GGTA1-transfected group was 41.04 ± 7.84, compared with 89.02 ± 5.60 ($P < 0.001$) in the immortalized cells and 82.62 ± 6.91 ($P < 0.001$) in the mock-transfected cells (Fig. 6B). Besides, as C3 consists of α-chain and β-chain and when C3 is activated, C3a would be cleaved from α-chain and α-chain could not be detected by anti-C3a antibody [13], we examined the C3 activation. As shown by Figure 6C, although all β-chains could be seen, α-chain of both immortalized cells and mock-transfected cells had disappeared. But in supernatant from our transfected cells, α-chain could be seen, although the color of the band α-chain in supernatant from the transfected cells is lighter than the color of the band that from human serum. These indicated that complements in HS incubated with our transfected cells cannot bind and activate as effectively as those in immortalized cells or mock-transfected cells.

4. Discussion

Interspecific molecular difference is the fundamental reason for xenograft rejection. Lessening these differences through transgenic technology is the most promising approach to solve this problem. With the development of GGTA1-knockout approaches, many different lines of transgenic pigs have become available. In 2006, Deppenmeier *et al.* developed pig lines carrying the GGTA1 knockout alone or combined with transfected human CD46, CD55, or CD59 [14]. More

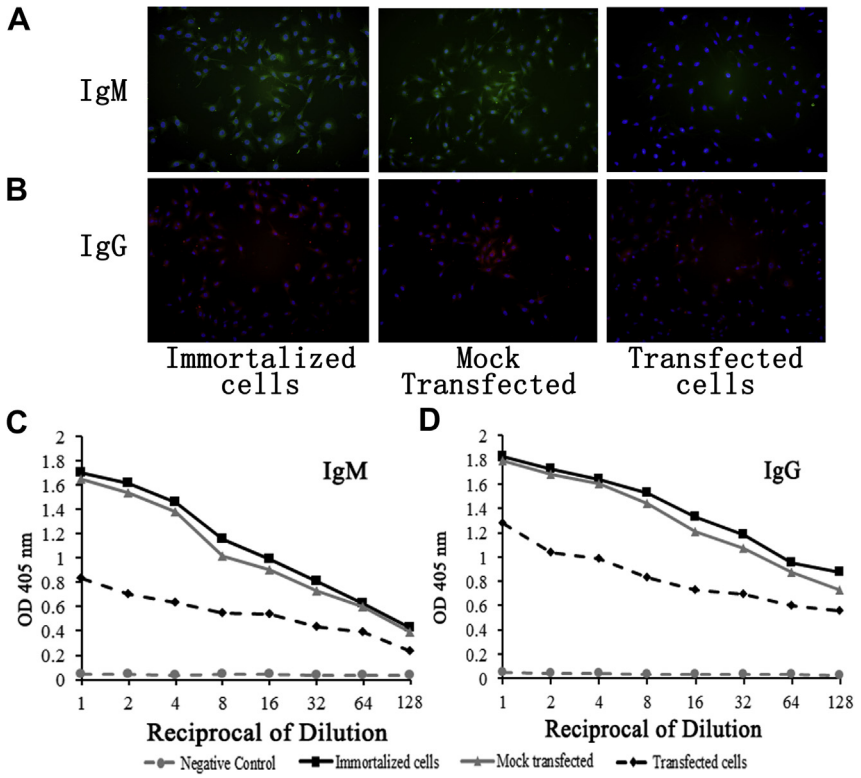

Fig. 5 – (A,B) Binding of human IgM (A) or IgG (B) on different cells by immunofluorescence. (C,D) Binding of human IgM (C) or IgG (D) in a series of dilution HS by ELISA. (Color version of figure is available online.)

recently, pig lines have been established that express porcine CTLA4-Ig [15], porcine retrovirus siRNA [16], HLA-E/human β2-microglobulin [17], human FAS ligand, or human thrombomodulin [18]. But their development and maintenance is relatively complicated, difficult, and expensive, thus restricting their usefulness in mass screening for molecules involved in rejection. These restrict the application of transgenic pigs in basic studies of xenotransplantation, making the development of cell culture–based models an urgent priority.

With the help of cell models, molecular interactions between pig and human tissues can be screened and the basic molecular mechanisms in xenograft rejection and the formation of a physiological barrier during transplantation can be confirmed. In 2008, Lee *et al.* [19] found that recombinant human tissue factor can interact with PAECs in culture, and this provided new strategies to address post-transplant coagulation disorders. Similarly, we expect that our cell culture model will identify key problems in xenograft reaction and reveal potential solutions quickly, compared to the long growth period necessary for transgenic animals. The establishment of additional cell models for xenotransplantation will improve the efficiency of identifying important molecules involved in transplantation-related immune pathology and potentiate further animal studies.

Factors in recipients' blood can lead to graft damage and rejection immediately after either allotransplantation or xenotransplantation. Endothelial cells modulate coagulation and are clearly involved in immune rejection. After porcine organs are transplanted into primates, blood-borne antibodies bind to the pig endothelial cells, mainly through α-1,3Gal. This binding activates the host complement system and results in production of membrane attack complexes, greatly increasing the possibility of intravascular thrombosis in the graft. Damaged graft endothelial cells themselves express many immune molecules that further increase the possibility of rejection and affect graft function [20,21]. Consequently, recent studies have mainly focused on the mechanisms of vascular injury and its mitigation using endothelial models. Because of this, we established a new GTKO PAEC cell line. We interfered with GGTA1 by RNAi to decrease the abundance of α-1,3Gal. RNAi clearly reduced the inhibition of cell proliferation and HS-induced apoptosis. IgG, IgM, and the complement system proteins C3 and C5b-9 did not bind as well to the α-1,3Gal-deficient cells and C3 activation was also reduced.

Although GGTA1 interference in our cells reduced immune molecular reactivity after exposure to HS, its effect was still greater in cells cultured with FBS. In HS, reactivation of C3 and binding of IgM, IgG, C3, and C5b-9 to the GGTA1-knockout cells was reduced relative to the controls but not completely eliminated. These results indicate that α-1,3Gal is not the only antigen involved in xenograft rejection. Non-Gal antigens also take part in the rejection. Galili *et al.* [22] analyzed the serum of graft recipients and found that antibodies to non-α-1,3Gal antigens were slower to appear than those to α-1,3Gal and conventional immunosuppressive strategies were less effective on non-α-1,3Gal antibodies. Rood *et al.* [23] found that antibodies to α-1,3Gal emerged 3 mo after birth in orangutans and increased with age, but antibodies to non-α-1,3Gal antigens were nearly undetectable 1 y after birth. However, the

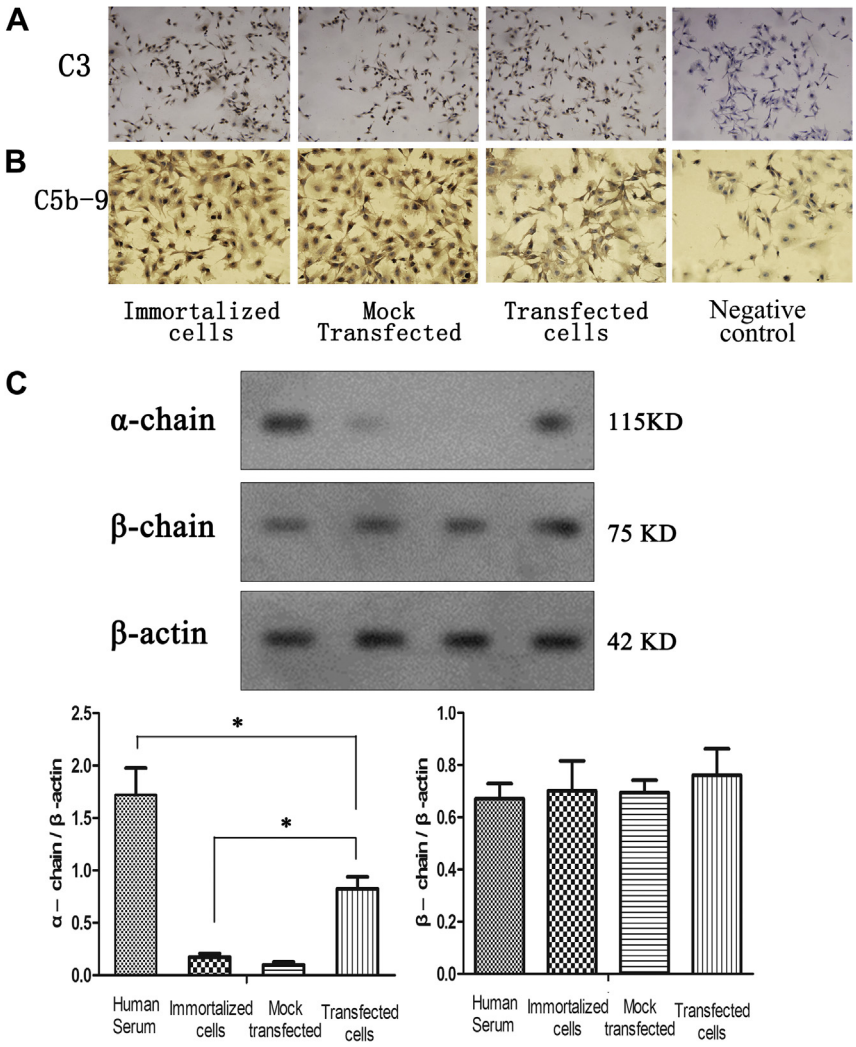

Fig. 6 – (A,B) Binding of human C3 (A) and C5b-9 (B) on different cells. (C) Western blotting analysis of C3 activation using monoclonal antibodies specific for C3 α-chain and β-chain. The signal intensities in the blot were assessed by densitometry and are represented in the graphs. One representation of three independent experiments is shown. *P < 0.01. (Color version of figure is available online.)

identity of these non-α-1,3Gal antigens and their role in xenograft rejection remain unknown. We believe that our cell model will also be useful in this field.

It should be noted that our current cell line and culture system are not perfect, and its application in xenotransplantation studies is limited. In this system, only a few immune molecules are mentioned. Immune cells such as T cells, B cells, natural killer cells, dendritic cells, and others are not well represented, and thus only immune responses that rely on natural antibodies and complement system proteins can be modulated. Studies have shown that natural killer cell–mediated cytotoxicity and innate immunity–induced graft injury still occur in GGTA1-knockout cells [24]. Current immunosuppressive strategies in allotransplantation have also been shown to be ineffective at preventing xenograft rejection. In addition, T and B cells likely have different reaction mechanisms in allotransplantation and xenotransplantation. For these reasons, we will continue to improve our cell and model system by incorporating more cell types and other immune factors so that we can simulate acute xenograft rejection in the future.

Acknowledgment

We thank Xia Li and Fuqin Zhang for their help in immunocytochemistry or immunofluorescence and flow cytometry.

The experiment was supported by National High Technology Research and Development Program (863 Program) (2012AA021005) and National Natural Science Foundation (81270549).

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