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Biocompatibility of Genetically-Engineered Pig Cornea in Corneal Xenotransplantation: Preliminary In Vitro Study

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Keywords: α 1,3-galactosyltransferase (GGTA1) | β 1,4-N-acetylgalactosaminyltransferase (β 4GalNT2) | corneal endothelial cell | corneal xenotransplantation | cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) | genetically engineered pig | thrombomodulin

ABSTRACT

Introduction: We aimed to evaluate biophysical compatibility of corneal endothelial cells in genetically-engineered (GE) pigs to identify the best candidate for in vivo nonhuman primate (NHP) study and to collect baseline data for future clinical trials from a biological function perspective.

Methods: Triple or quadruple knock-out (T[Q]KO) pigs with inactivation of α 1,3-galactosyltransferase (GGTA1), cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), β 1,4-N-acetylgalactosaminyltransferase (β 4GalNT2), and/or isoglobotrihexosylceramide synthase (iGB3s) genes were used in combination with insertion of Human(*h*) CD55, CD39, CD46, and thrombomodulin (TBM) genes. Twenty-seven eyeballs obtained from 14 GE pigs were used to evaluate the physical parameters and functional properties as a suitable donor. Corneal endothelial cell density (ECD) was serially measured for 7 days in a preservative solution. Doubling time (DT) for EC proliferation and immunofluorescence staining of corneal endothelial pump channels and tight junctional proteins were evaluated.

Results: Mean age of GE pigs was 11.9 months old and mean central corneal thickness was 718.2 μ m. Mean ECD loss/week was significantly higher in GE pigs younger than 6 months, which was 55.1%, while it was 8.8% in GE pigs older than 6 months ($p < 0.001$). T(Q)KO/*h*CD46/*h*TBM cornea showed early severe apoptotic or necrotic changes of endothelial cells, whereas other GE corneas did not. Although the mean DT increased in all GE pigs, it showed no difference between the younger and older groups. ZO-1, N-cadherin, ATPase, SLC4A1, and aquaporin 1 were well expressed on endothelial cells regardless of modified gene types.

Conclusion: It suggests that no effect of the added gene was observed on the endothelial cell functional capacities in TKO(QKO) pig corneas, and GE pigs over 6 months old are suitable as corneal transplant donors.

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1 | Introduction

The concept of corneal transplantation to replace an opaque human cornea with a transparent animal cornea was first suggested by Franz Riesinger in 1824 [1, 2]. Even though the first reported pig-to-human corneal xenotransplantation was done in 1838 [3], decades before the first attempt of corneal allotransplantation in 1905 [4], xenotransplantation paved the way to allotransplantation due to insurmountable xenogeneic immune reaction in the past. Recently, the advent of genetically engineered (GE) pigs, especially alpha-1,3-galactosyltransferase gene-knockout (GT-KO) pigs, which aimed to reduce hyperacute rejection [5, 6], brought xenotransplantation a new era of possibility. Also, the latest gene-editing technology of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 made fast and successful multiple gene editing possible and enabled porcine endogenous retrovirus (PERV) inactivation, eliminating the risk of xeno-zoonosis [7]. Owing to those, xenotransplantation of multiple gene-edited porcine organs to humans became a reality, and clinical trials of porcine heart, liver, and kidney xenotransplantation to humans had been reported in the present [8–10].

Cornea donor shortage, especially in Eastern Asia, due to religious and cultural reasons, has demanded new donor candidates for corneal transplantation [2]. Pre-clinical studies of pig-to-rhesus penetrating keratoplasty (PKP) with various immunosuppressants were proven successful, PKP with an anti-CD154 antibody regimen with a 100% survival rate for 6 months [11], anti-CD40 antibody with 83.3% [12], and anti-CD20 antibody with 71.4% [12]. Initially, one or two gene-edited porcine corneas did not show a significant increase in graft survival compared to wild-type porcine corneas without heavy immunosuppressants [2, 13, 14]. To overcome the hurdle of the demand for heavy immunosuppressants, multiple gene-edited pigs have been still developing up to date as a candidate for the use of human clinical trials [15]. However, it has not been evaluated so far whether the corneal endothelial cell function would remain intact when the multiple genes related to immune reactions are edited in pigs.

Therefore, this study aimed to identify the best GE pig candidate for in vivo nonhuman primate (NHP) corneal xenotransplantation and to collect baseline data for future clinical trials in terms of biological function. Since endothelial cells are crucial in maintaining corneal transparency in full-thickness corneal xenotransplantation, we mainly focused on the characteristics of corneal endothelial cells of various GE pigs.

2 | Materials and Methods

All study procedures followed the ARVO statement regarding the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Research Ethics Committee at Seoul National University Hospital (Institutional Animal Care and Use Committee, IACUC No. 23-0296-S1A0). Human cornea data as a positive control for comparison in Immunofluorescence staining were collected following the approval of the Institutional Review Board of Seoul National University Hospital (IRB No. H-2308-049-1456).

All GE Pigs were provided by Optipharm Inc. (Chungcheongbuk-do, Korea). Target genes for Knock-out (KO) to reduce humoral responses were as follows [16]: α 1,3-galactosyltransferase (GGTA1), isoglobotrihexosylceramide synthase (iGB3s), Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), and β 1,4-N-acetylgalactosaminyltransferase (β 4GalNT2) KO. Yucatan miniature pigs with GGTA1/CMAH/ β 4GalNT2 triple knockout (TKO) were produced by using the CRISPR/Cas9 system, as previously described [16]. Also, Optipharm Inc. was granted a patent for producing GGTA1/CMAH/ β 4GalNT2/iGB3s quadruple knockout (QKO) pigs (National patent application number: 102040203), which were further edited to eliminate the iGB3s gene using the CRISPR/Cas9 system, as mentioned above. Genetic deficiency of iGB3s can reduce recognition of iGB3 by NKT cells [17]. The ablation of GGTA1, CMAH, and β 4GalNT2 was confirmed in peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS) technique (data not shown), while iGB3s knockout had not been tested yet due to the lack of an applicable antibody.

Genes for *hCD55* (human decay-accelerating factor) and *hCD39* (human Ectonucleoside triphosphate diphosphohydrolase) were knocked-in to inhibit complement activation and coagulopathy [18]. cDNA for *hCD55* and *hCD39* were inserted under the control of the GGTA1 endogenous promoter in exon 4 of the GGTA1 gene locus, as previously described [18]. Expression of *hCD55* and *hCD39* on corneal endothelial cell surface as well as PBMCs was confirmed in the previous study [18].

To intervene in complement activation [15], genes for *hCD46* (human membrane cofactor protein) and *hTBM* (human thrombomodulin) were inserted by the method previously described [19]. In brief, it is exogenous promoter-mediated expression of genes [19]. Kimyung Choi constructed the pCAGGS-*hCD46*-2A-*hTBM* vector, which expresses human CD46 and human TBM linked by a 2A peptide under the control of the CMV early enhancer/chicken β -actin promoter (pCAGGS) [19]. The constructed vectors were transfected into Yucatan miniature pig fibroblasts by electroporation [19]. Verified transgenic cells were then used in Somatic cell nuclear transfer (SCNT) to produce GTKO/*hCD46*/*hTBM* pigs [19]. Expression of *hCD46* and *hTBM* on corneal endothelial cells was confirmed using FACS (data not shown). Twenty-seven eyes of 14 genetically-engineered (GE) pigs were analyzed. GE pigs were grouped into three groups according to genetic modifications. Triple gene (GGTA1/ β 4galNT2/CMAH) knock-out (TKO) and quadruple gene (GGTA1/ β 4galNT2/CMAH/iGB3s) knock-out (QKO) with the combinations of selected human genes (*hCD55*, *hCD39*, *hCD46*, and human thrombomodulin, *hTBM*) insertion were designated for GE pig production. GE cornea was divided into three groups as follows ($n = 14$); T(Q)KO/*hCD55*/*hCD39*, QKO, and T(Q)KO/*hCD46*/*hTBM* groups; For each group, five pigs were included in T(Q)KO/*hCD55*/*hCD39* group, six in QKO group, and three in T(Q)KO/*hCD46*/*hTBM* group, as described in Table 1. T(Q)KO/*hCD55*/*hCD39* group included one QKO/*hCD55*/*hCD39* pig (#13) and four TKO/*hCD55*/*hCD39* pigs, while T(Q)KO/*hCD46*/*hTBM* group included one TKO/*hCD46*/*hTBM* pig (#10) and two QKO/*hCD46*/*hTBM* pigs. This grouping, which includes heterogeneous genotypes, was intended solely to assess biological endothelial cell function, not immunological response.

TABLE 1 | Demographic, physical, and optical properties of genetically engineered pigs and their corneas

Gene modification	No.	Age (months)	Sex	BW (kg)	HCD (mm)	VCD (mm)	CCT ^b (μm)
T(Q)KO/ <i>hCD55/hCD39</i>	1	3.3	M	9	11.8 ± 0.1	11.2 ± 0.4	—
	2	2	M	8.6	12.7 ± 0.2	11.0 ± 0.0	—
	5	2.8	F	9.5	12.1 ± 0.1	11.1 ± 0.1	764.5 ± 15.5
	13 ^a	16.0	M	69	17.0 ± 0.0	15.5 ± 0.0	794.0 ± 22.0
	14	15.8	F	41.5	14.9 ± 0.1	14.1 ± 0.1	851.5 ± 17.5
	Mean ± SD	8.0 ± 6.5	—	27.5 ± 24.3	13.7 ± 2.0	12.6 ± 1.9	803.3 ± 40.6
QKO	3	33.7	M	91	17.6 ± 0.2	16.8 ± 0.3	684.5 ± 55.5
	4	3.6	M	13.5	13.7 ± 0.2	11.9 ± 0.1	—
	6	30.2	F	71.5	17.0 ± 0.0	15.5 ± 0.0	646.5 ± 7.5
	7	17.3	F	63	16.3 ± 0.3	14.8 ± 0.3	615.5 ± 15.5
	8	19.2	M	67.5	16.2 ± 0.2	15.0 ± 0.0	760.5 ± 84.5
	9	16.0	M	60.5	16.5 ± 0.0	15.3 ± 0.3	620.5 ± 34.5
	Mean ± SD	20.0 ± 9.9	—	61.2 ± 23.5	16.2 ± 1.2	14.9 ± 1.5	665.5 ± 72.1
T(Q)KO/ <i>hCD46/hTBM</i>	10	2.3	M	7	12.2	10.9	753
	11	2.2	F	7	12.1 ± 0.1	10.4 ± 0.1	Undetectable
	12	1.8	M	5.5	11.3 ± 0.1	11.0 ± 0.1	708.5 ± 4.5
	Mean ± SD	2.1 ± 0.2	—	6.5 ± 0.7	11.8 ± 0.4	10.7 ± 0.3	723.3 ± 21.3
SNU miniature pig cornea ^c (previous study)	—	41.7 ± 18.7	M(37), F(14)	82.4 ± 17.1	17.1 ± 0.4	15.5 ± 0.5	852.6 ± 65.2

Note: HCD, VCD, and CCT values are the mean values of two eyes of each individual. Exceptionally, only one eye was included from pig #10. Values are shown as mean ± standard deviation (SD).

Abbreviations: α1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:IGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β1,4-N-acetylgalactosaminyltransferase:β4GalNT2; BW, body weight; CCT, central corneal thickness, *hTBM*, human thrombomodulin; HCD, horizontal corneal diameter; KO, knock-out; QKO, quadruple (GGTA1/B4galNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/B4galNT2/CMAH) KO; VCD, vertical corneal diameter.

^aBoth corneas from pig #13 were separately used for pig-to-rhesus corneal penetrating keratoplasty, so these corneas were not further studied for serial changes of endothelial cell count.

^bCCT was measured either after extraction of eyes (T(Q)KO/*hCD55/hCD39KI*, T(Q)KO/*hCD46/hTBM*) or in vivo for QKO.

^cData are from the previous study, Kim DH, et al. Biophysics-functional compatibility of Seoul National University (SNU) miniature pig cornea as xenocorneal graft for the use of human clinical trial. Xenotransplantation. 2016;23(3):202-210.

2.1 | Physical and Optical Properties of GE Pig Cornea

After enucleation of the eyes, corneal parameters were examined before corneoscleral rim excision. Vertical and horizontal corneal diameters were measured with the Castroviejo caliper. Central corneal thickness (CCT) was measured by an ultrasonic pachymeter (Quantel Medical, Clermont-Ferrand, France). CCT was measured either after the extraction of the eye within 5 h (T(Q)KO/*hCD55/hCD39*, T(Q)KO/*hCD46/hTBM*) or in vivo before enucleation for QKO. The mean value of corneal thickness of both eyes was used in each GE pig. Optical properties of QKO/*hCD55/hCD39* and QKO pigs were analyzed with anterior segment optical coherence tomography (AS-OCT; Visante, Carl Zeiss Meditec, Dublin, CA, USA). Steep and flat keratometry data were obtained.

2.2 | Changes of Corneal Endothelial Cell Density (ECD) in Preservative Solution

For use in clinical trials, the corneal graft can be preserved with a certified preservative solution up to 7 days. Corneoscleral rim excision of enucleated eyes was done within 5 h after enucleation. Corneas with peripheral scleral rim were stored in Optisol GS (Bausch+Lomb, Irvine, CA, USA), which is the commonly utilized Food and Drug Administration (FDA)-approved preservation media for cornea. Within the media, endothelial cell density (ECD) was measured by a confocal microscope using Konan Eye Bank KeratoAnalyzer (EKA-10, Konan, Japan). ECD was measured daily until Day 7 of enucleation, and serial changes in ECD were analyzed from Day 0 to Day 7. Initial ECD, final ECD, and mean ECD loss (%) for 1 week were compared between groups based on various GE pigs.

2.3 | Corneal Endothelial Cell Proliferation

Dissected corneas from corneoscleral rims of eyes were used for endothelial cell harvest. Endothelial cells were harvested, centrifuged, and cultured following the method successfully used for pig corneal endothelial cell proliferation in the previous study [20]. Corneal endothelial cells from the first passage were incubated in 100 mm culture dishes (2.0×10^5 cells/dish). The number of endothelial cells was counted every 2 days until 21 days. Doubling time was calculated as described by Fujita et al. [21] [doubling time = $t/10(\log D_2 - D_1)$] and Konomi et al. [22] [doubling time = $(t_1 - t_2) \log 2 / (\log N_2 - \log N_1)$]. Cell number was counted between about Day 5 and Day 12.

2.4 | Corneal Endothelial Cell Function for Tight Junction and Water-Pump

After 7 days of Optisol GS preservation, corneal tissues were fixed in 10% neutral buffered formalin for 24 h. Immunofluorescence staining of barrier function with tight junction (ZO-1, N-cadherin) and water-pump channels (ATPase, aquaporin 1, SLC4A1) of endothelial cells was conducted. Antibodies used were as follows: ZO-1 (1:100; Invitrogen, Austin, TX), N-cadherin (1:100; ProteintechGroup, Rosemont, IL), ATPase (1:100; Abcam, Cambridge, MA), Aquaporin 1 (1:100; ProteintechGroup, Rosemont, IL), SLC4A1 (1:100; Novus Biologicals, Centennial, CO). The photographs of immunofluorescence staining in each tissue were taken using confocal microscopy (Konan Eye Bank KeratoAnalyzer, EKA-10, Konan, Japan) and were documented with high-power magnification ($\times 200$). For ATPase and SLC4A1 immunofluorescence staining, the human cornea was used as a positive control.

Data of SNU miniature pigs [20], whose corneas were proven successful in pig-to-rhesus corneal xenotransplantation, were used as a reference to compare with data of GE pig cornea in evaluation for optical properties and characteristics of corneal endothelial cells.

3 | Results

3.1 | Physical and Optical Properties of GE Pig Cornea

Demographics of GE pigs and their corneal physical and optical properties are summarized in Table 1. All pigs of the T(Q)KO/hCD46/hTBM groups were younger than 6 months. In the T(Q)KO/hCD55/hCD39 group, pig #13 was 16 months old and pig #14 was 15.8 months old, while the other three TKO/hCD55/hCD39 pigs were younger than 6 months. In QKO group, one pig (#4) was 3.6 months old, while the other five pigs were older than 6 months. Mean horizontal and vertical corneal diameter ranged from 10.4 to 13.7 mm in pigs younger than 6 months and from 14.1 to 17.6 mm in pigs older than 6 months. These were comparable with SNU pig cornea data, which showed a mean horizontal diameter of 17.1 ± 0.4 mm and a mean vertical diameter of 15.5 ± 0.5 mm at the mean age of 41 months. Mean central corneal thickness was variable from 665.5 ± 70.1 (in vivo) to 851.5 ± 17.5 μ m (after death), depending on GE types and age,

but it was not thicker than the SNU miniature pig cornea, with the mean value of 852.6 ± 65.2 μ m, which had already been proven to be surgically operable.

In Table 2, optical properties of corneas from T(Q)KO/hCD55/hCD39 pigs and QKO pigs, both with the age older than 6 months, were compared. Both corneas of T(Q)KO/hCD55/hCD39 pig (40.3D) and QKO pig (38.3D) showed slightly steeper refractive power when compared with SNU miniature pig corneal refractive power (36.3D). Average CCT of QKO pig corneas, which were measured in vivo, showed the thinnest thickness of 678.0 μ m, whereas average CCT of T(Q)KO/hCD55/hCD39 pig corneas, which were measured after enucleation, was 822.8 μ m. Nevertheless, the average CCT of both T(Q)KO/hCD55/hCD39 and QKO pig was thinner than the CCT of SNU miniature pig (852.6 μ m) which was measured after enucleation. Initial average corneal endothelial cell density was higher in both GE pigs showing that T(Q)KO/hCD55/hCD39 pig of 3109/mm² and QKO pig of 2883/mm², when compared with SNU miniature pig cornea of 2625/mm².

3.2 | Changes of Corneal Endothelial Cell Density (ECD)

Figure 1 contains confocal microscopic images at Day 0 and Day 7 of the corneal endothelium of different GE pigs. First row images (A, B, C, D) were taken on the initial day, and the second row images (E, F, G, H) at Day 7. Cornea endothelial images of 2.8-month-old TKO/hCD55/hCD39 pig (pig #5) are in A and E, QKO pig of 33.7 months (pig #3) on B and F, QKO pig of 3.6 months (pig #4) on C and G, TKO/hCD46/hTBM pig of 2.3 months (pig #10) on D and H. Initial endothelial cells were smaller and cell density was higher in pigs with younger age (A, C and D) regardless of gene modification type, but ECD was decreased due to partial apoptotic or necrotic changes of the endothelial cells (red arrows) at Day 7 in younger age whereas ECD was well maintained between Day 0 and Day 7 in the other older pig (Figure 1B,F). Meanwhile, early guttata-like apoptotic changes were already observed at Day 0 in TKO/hCD46/hTBM cornea, resulting in severe apoptotic or necrotic changes of the endothelial cells at Day 7 (Figures 1D, H, red arrows).

Quantitative analysis of serial changes of mean ECD in preservative solutions until 7 days is summarized in Figure 2 and Table 3. All pigs in the T(Q)KO/hCD46/hTBM group were under 6 months. In TKO/hCD55/hCD39, T(Q)KO/hCD46/hTBM, and in QKO group with age younger than 6 months, initial ECD was high (5403 ± 218 , 6216 ± 457 , and 5417 ± 243 /mm² each). However, endothelial cell loss (ECL) for 1 week was also high, which ranged from 38.93% to 61.70%. Whereas, in the TKO/hCD55/hCD39 and QKO group with age older than 6 months, initial mean ECD was well maintained until Day 7, with the acceptable mean ECL for 1 week of 11.1% and 10.5% for each. It was comparable with SNU miniature pigs whose initial mean ECD was 2625 ± 81 /mm² and mean ECL was $7.6 \pm 1.5\%$. In Figure 2B and Table 3, when the pigs were re-grouped based on their age with the cut-off value of 6 months, ECD was significantly higher in younger age at Day 0 ($p < 0.001$, Mann-Whitney Test) until Day 3 ($p = 0.031$, Mann-Whitney Test), but there became no significant difference of ECD at Day 7 because of high ECL in younger age group. Mean ECL for

TABLE 2 | Optical properties of genetically engineered pig cornea

	T(Q)KO/ <i>h</i> CD55/ <i>h</i> CD39 pig ^a	QKO pig ^b	SNU miniature pigs ^c
Age (months)	15.9 ± 0.1	24.8 ± 7.4	41.7
Steep Keratometry (D)	40.7 ± 1.4	39.3 ± 2.0	37.4 ± 1.8
Flat Keratometry (D)	39.8 ± 1.0	37.2 ± 1.7	35.2 ± 1.5
Central corneal thickness (ultrasound pachymetry, μm)	822.8 ± 35.0	678.0 ± 75.2	852.6 ± 65.2
Endothelial cell density (cell/mm ²)	3109 ± 34	2883 ± 245	2625 ± 81

Note: Values are shown as mean ± standard deviation (SD).
Abbreviations: α1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β1,4-N-acetylgalactosaminyltransferase:β4GalNT2; KO, knock-out; QKO, quadruple (GGTA1/B4galNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/B4galNT2/CMAH) KO.
^aT(Q)KO/*h*CD55/*h*CD39 pig included pig #13 and #14.
^bQKO pig data included data of pig #3,6, 8,9.
^cData are from the previous study, Kim DH, et al. Xenotransplantation. 2016;23(3):202-210.

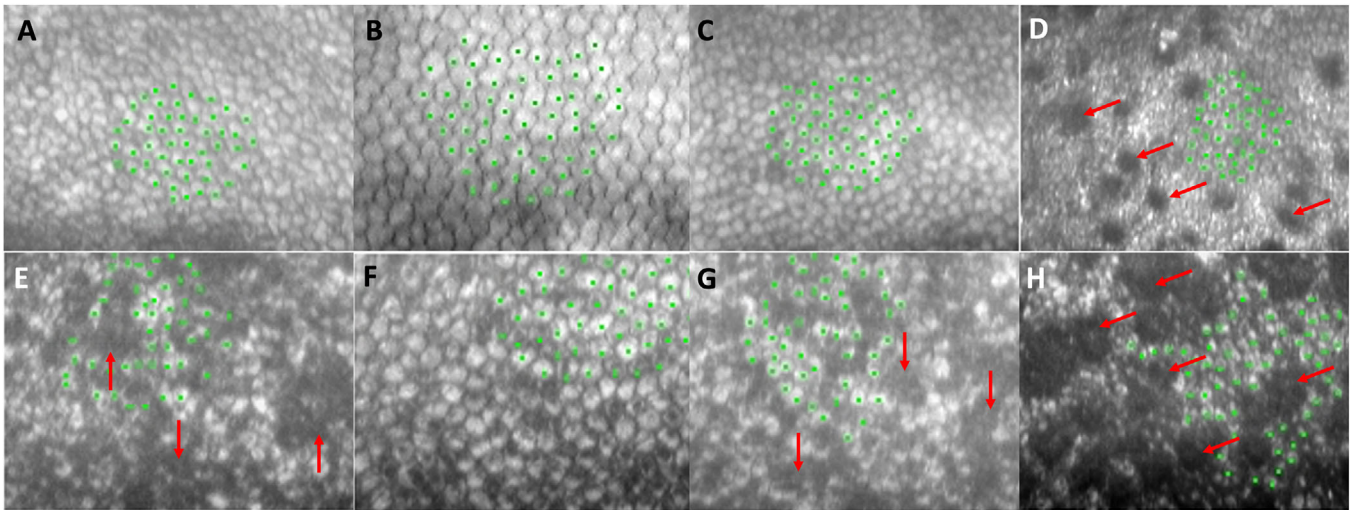


FIGURE 1 | Confocal microscopy images of genetically-engineered (GE) pig corneal endothelium at Day 0 and Day 7. Confocal microscopy images of GE pig corneal endothelium preserved in Optisol solution, taken at the initial time point and on Day 7, are shown. First row images (A, B, C, D) were taken at Day 0, and the second-row images (E, F, G, H) at Day 7. A,E: TKO/*h*CD55/*h*CD39 pig of 2.8 months old (pig #5), B,F: QKO pig of 33.7 months old (pig #3), C,G: QKO pig of 3.6 months old (pig #4), D,H: TKO/*h*CD46/*h*TBM pig of 2.3 months old (pig #10). Apoptotic or necrotic changes of the endothelial cells are marked with red arrows. α1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β1,4-N-acetylgalactosaminyltransferase:β4GalNT2; *h*TBM, human thrombomodulin; KO, knock-out; QKO, quadruple (GGTA1/B4galNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/B4galNT2/CMAH) KO.

1 week was significantly lower in the older age group, which was 8.8 ± 4.8%, compared with 55.1 ± 4.1% of the younger age group ($p < 0.001$, Mann-Whitney Test).

3.3 | Corneal Endothelial Cell Proliferation

Cell count results of corneal endothelial cell culture are shown in Figure 3. Corneal endothelial cells showed proliferative capacities in all groups. In Table 4, the calculated doubling time (DT) of the corneal endothelial cells of the three groups is summarized. Mean DT of TKO/*h*CD55/*h*CD39 pigs were 81.9 and 100.9 h with different doubling time calculation methods, 73.5 and 139 h in QKO pigs, and 72.2 and 113.8 h in T(Q)KO/*h*CD46/*h*TBM pigs, respectively. DT of all GE groups, ranging from 72.2 to 139.0 h,

was longer than that of SNU miniature pigs, which was calculated to be from 50.5 to 54.9 h depending on different calculation methods. When we looked at the age effect on DT. There were no differences in DT between the two age groups, as the younger age group had 74.0 to 103.6 h and the older age group had 75.2 to 143.1 h. ($p = 0.647$ and 0.361 each with different doubling time calculation methods, Mann-Whitney Test).

3.4 | Corneal Endothelial Cell Function

Immunofluorescence staining images of the corneal endothelial cells' barrier and pump channels are shown in Figure 4. Two representative barrier channels, ZO-1 and N-Cadherin, were well-stained in all groups. Also, antibodies of three pump channels,

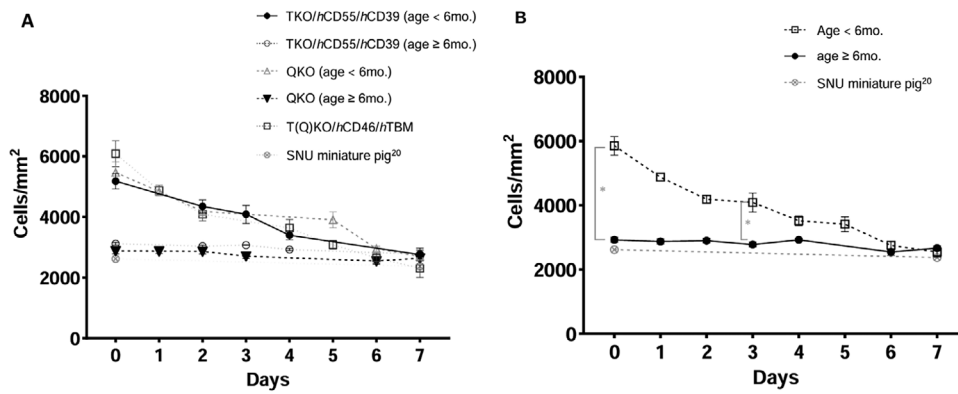


FIGURE 2 | Serial changes in corneal endothelial cell density of genetically engineered pigs in preservative solution after enucleation. In A, groups are divided based on gene modification types, and in B, groups are divided based on age [20]. Data from the previous study are included as comparable control data. *Mann-Whitney test between groups done. (p value < 0.05 considered significant) values are shown with mean $\pm$ standard error (SE). α 1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β 1,4-N-acetylgalactosaminyltransferase: β 4GalNT2; hTBM, human thrombomodulin; KO, knock-out; QKO, quadruple (GGTA1/B4galNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/B4galNT2/CMAH) KO.

TABLE 3 | Comparison of corneal endothelial cell density (ECD) and mean ECD loss based on types of genetic modification and age

	TKO/hCD55/hCD39 (age < 6 mo.)	TKO/hCD55/hCD39 (age $\geq$ 6 mo.)	QKO (age < 6 mo.)	QKO (age $\geq$ 6 mo.)	T(Q)KO/hCD46 /hTBM
ECD at D0	5403 $\pm$ 218	3121 $\pm$ 44	5417 $\pm$ 243	2883 $\pm$ 87	6216 $\pm$ 457
ECD at D7	3337 $\pm$ 479	2777 $\pm$ 131	2701 $\pm$ 194	2575 $\pm$ 57	2288 $\pm$ 187
ECL (%)	38.9 $\pm$ 11.1	11.1 $\pm$ 3.0	50.1 $\pm$ 5.8	10.5 $\pm$ 1.0	61.7 $\pm$ 5.2
	Age < 6 mo.		Age $\geq$ 6 mo.		p value ^a
ECD at D0	5853 $\pm$ 288		2930 $\pm$ 253		< 0.001
ECD at D3	4087 $\pm$ 299		2785 $\pm$ 240		0.031
ECD at D7	2553 $\pm$ 162		2672 $\pm$ 240		0.887
ECL (%)	55.1 $\pm$ 4.1		8.8 $\pm$ 4.8		< 0.001

Note: Values are shown with mean $\pm$ standard error (SE). Endothelial cell density (ECD, cells/mm²) at Day (D) 0, Day 3, and Day 7 after corneoscleral rim excision. Abbreviations: α 1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β 1,4-N-acetylgalactosaminyltransferase: β 4GalNT2; ECL, mean endothelial cell density loss for 1 week; hTBM, human thrombomodulin, KO, knock-out; QKO, quadruple (GGTA1/B4galNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/B4galNT2/CMAH) KO.

^aMann-Whitney Test (p value < 0.05 considered significant).

ATPase, AQP1, and SLC4A11, were shown to be positive in all groups. These results indirectly supported preserved corneal endothelial cell pump functions and maintained tight junction barrier in GE pig corneas.

4 | Discussion

This study shows that both T(Q)KO/hCD55/hCD39 and QKO pig corneas are of acceptable endothelial cell (EC) functional capacities in pigs older than 6 months old as a suitable donor for human corneal xenotransplantation. Although GE pig corneas were larger and thicker than human corneas [23], they were smaller and thinner than those of the SNU miniature pig [20]. The mean refractive power of T(Q)KO/hCD55/hCD39 and QKO pig corneas was steeper than that of the SNU miniature pig cornea. Given that SNU miniature pig corneas were well-functioned after transplan-

tation into Non-human primates, T(Q)KO/hCD55/hCD39 and QKO pig corneas with older ages above 6 months also seem to be biocompatible for transplantation. Early severe guttatic changes of T(Q)KO/hCD46/hTBM or huge ECL of younger GE pigs (< 6 months) in preservative solution up to 7 days indicate that they may not be compatible for the use in a clinical trial for full-thickness corneal transplantation (i.e., penetrating keratoplasty).

At first, we looked at the corneal thickness to evaluate whether the GE pig cornea would be surgically operable. Average CCTs of GE pigs in this study were comparable to or even thinner (QKO) than the mean CCTs in wild-type pigs (Wally Whippo, Enon Valley, PA, USA) of the age of 5–10 months with 775.4 $\pm$ 47.1 μ m [24], GTKO/hCD46/ $\pm$ hCD55 pigs (Revivicor, Blacksburg, VA, USA) of 15 months with 867.9 $\pm$ 3.3 μ m [24] or SNU miniature pig, whose mean age was 41.7 months, with 852.6 $\pm$ 65.2 μ m. Since human

TABLE 4 | Doubling time of corneal endothelial cells in different groups based on types of genetic modification and age

	TKO/ <i>hCD55</i> / <i>hCD39</i>	QKO	T(Q)KO/ <i>hCD46</i> / <i>hTBM</i>
Doubling time (a, hours)	81.9	73.5 ± 7.8	72.2 ± 5.3
Doubling time (b, hours)	100.9	139.0 ± 62.9	113.8 ± 10.0
	Age < 6 mo.	Age ≥ 6 mo.	<i>p</i> value ^a
Doubling time (a, hours)	74.0 ± 2.9	75.2 ± 2.9	0.647
Doubling time (b, hours)	103.6 ± 8.3	143.1 ± 26.5	0.361
SNU miniature pig ^b			
Doubling time (a, hours)	54.9		
Doubling time (b, hours)	50.5		

Note: Values are shown with mean ± standard error (SE).

Doubling time (a) = $t/10(\log D2 - D1)$.

Doubling time (b) = $(t1 - t2) \log 2 / (\log N2 - \log N1)$.

Abbreviations: α 1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β 1,4-N-acetylgalactosaminyltransferase: β 4GalNT2; *hTBM*, human thrombomodulin, KO, knock-out; QKO, quadruple (GGTA1/ β 4GalNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/ β 4GalNT2/CMAH) KO.

^aMann-Whitney Test (*p* value < 0.05 considered significant).

^bData are from the previous study, Kim DH, et al. Xenotransplantation. 2016;23(3):202-210.

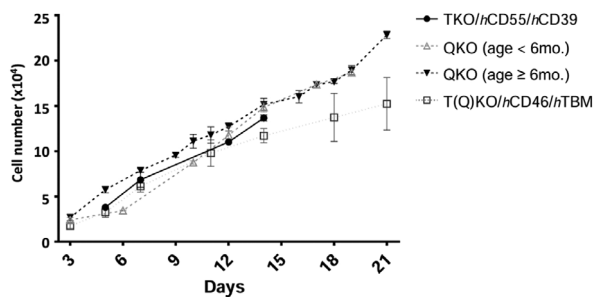


FIGURE 3 | Proliferative capacity of corneal endothelial cells of genetically-engineered pigs. Values are shown with mean ± standard error (SE) on the graph. α 1,3-galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, β 1,4-N-acetylgalactosaminyltransferase: β 4GalNT2; *hTBM*, human thrombomodulin; KO, knock-out; QKO, quadruple (GGTA1/ β 4GalNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/ β 4GalNT2/CMAH) KO.

corneal thickness is thinner than these pig corneas, CCT is 523 μ m and peripheral CT is 660 μ m, the thinner thickness of these GE pig corneas (665–803 μ m) of this study must be a favorable factor in approximation of the edge in corneal xenotransplantation rather than thicker cornea of the other GE pigs (775–867 μ m).

Second, we looked into the optical quality of refractive steepness of GE pigs, which is also comparable to that of SNU miniature pig [20]. Considering that appropriate donor-recipient size disparity during corneal transplantation could make the graft steeper than that of donor refractive power, although that the average human cornea is steeper (average to be 43.48D in a previous study [25]) than GE pig corneas, GE pig corneas also seem to be optically qualified for the use in clinical trials.

Different strategies have been adopted to avoid immune reactions in xenotransplantation [15]. To suppress hyperacute rejection, α 1,3-galactosyltransferase (GGTA1) and isoglobotrihexosylceramide synthase (iGB3s) were knocked out while human complement factor, *hCD46* (transgenic human membrane cofactor protein) and *hCD55* (transgenic human decay-accelerating factor) were inserted. For suppression of acute humoral xenograft rejection, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) and β 1,4-N-acetylgalactosaminyltransferase (β 4GalNT2) were knocked out. To reduce coagulation disorder, human Ectonucleoside triphosphate diphosphohydrolase (*hCD39*) and human thrombomodulin (*hTBM*) were inserted. Combinations of different gene alterations were adopted in the pigs used in this study. These days, initial steps have been taken with GE pig organs into humans. Heart from a GE pig with 10 genetic modifications [8], liver from a 6-gene-modified pig [9], and even kidney from 69-gene-modified pig [10] were used for xenotransplantation into humans with early success results. To minimize the use of immunosuppressive agents and therefore elongate graft survival, diverse gene modification combinations are now being studied. To be transparent, the graft, water-pump, and tight barrier of donor EC should be well-functioning with an appropriate EC density (ECD) after transplantation. Therefore, we are concerned whether multiple immune-related gene modifications would affect EC function and density.

To address this issue, we further evaluated the initial and dynamic functional capacities of GE pig corneal endothelial cells (EC). Surprisingly, GE pigs younger than 6 months showed initially high corneal ECD regardless of gene type variations at first. However, it went down very rapidly during preservation in younger age donors. Although initially high, a rapid drop in ECD is a critical issue for corneal transplantation. The abrupt loss of endothelial cells due to a large amount of apoptotic or necrotic cell death can trigger a severe inflammatory cascade,

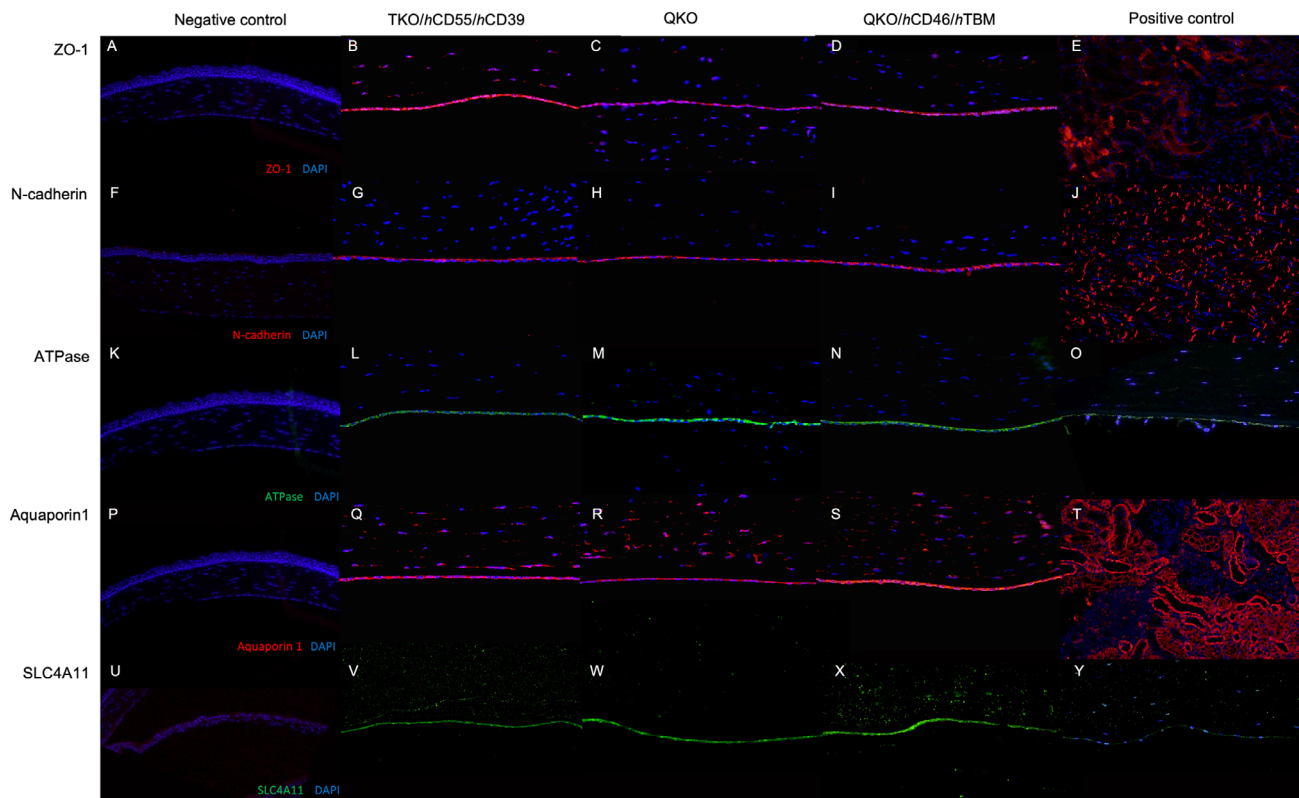


FIGURE 4 | Immunofluorescence staining of barrier proteins and pump channels in the corneal endothelial layer in genetically-engineered pigs. ZO-1, N-cadherin, and aquaporin 1 are stained in red, ATPase and SLC4A11 are stained in green. DAPI is shown in blue. Image magnification was $\times 400$. $\alpha 1,3$ -galactosyltransferase:GGTA1, isoglobotrihexosylceramide synthase:iGB3s, cytidine monophosphate-N-acetylneuraminic acid hydroxylase:CMAH, $\beta 1,4$ -N-acetylgalactosaminyltransferase: $\beta 4$ GalNT2; *hTBM*, human thrombomodulin; KO, knock-out; QKO, quadruple (GGTA1/B4galNT2/CMAH/iGB3s) KO; TKO, triple (GGTA1/B4galNT2/CMAH) KO.

exacerbating both innate immunity and xenogeneic responses. This study supports the previous research involving young GE pig donors, which showed an unexpectedly worse outcome after corneal xenotransplantation [13, 26]. Early studies with GE pigs for corneal xenotransplantation used very young donors because of the high disparity of corneal thickness issues [13, 26]. On the other hand, GE pigs older than 6 months showed comparable mean ECD loss of SNU miniature pig cornea [20] and that of healthy human, which was previously reported to be 10% in optisol GS solution [27]. It means that the age of the GE pig is relevant to maintaining endothelial cell stability. Therefore, pigs of any gene modification younger than 6 months would be reluctantly used for corneal transplantation due to significantly higher mean ECD loss. Given the relatively thin corneal thickness of these GE pigs and the less thickness disparity between donor and recipient, we can take advantage of older donor GE pigs (≥ 6 months) with low ECL for the use in clinical trials for corneal xenotransplantation.

As for endothelial cell proliferation capacity, corneal EC in GE pigs needed a longer doubling time than SNU miniature pig cornea. Although it is hard to compare directly with having different settings, DT in GE pigs in our study was also longer than DT calculated in previous GE pig corneas (GTKO/*hCD46*/ $\pm$ *hCD55*) or in human corneas [21]. We could not compare DT among various gene groups due to the small numbers in our study. Although there was no difference in DT between the two groups

depending on age, multiple gene modifications might have affected the proliferative capacity of ECs. When we evaluated the main function of corneal ECs, it looked compatible by showing good expression of major ion pumps and tight junction proteins in EC of GE pig cornea of all types in this study.

Interestingly, as shown in Figure 1D, pig corneas with human thrombomodulin gene insertion showed numerous guttata, which are markers of corneal endothelial cell dysfunction and loss, in the early stage of preservation. ECD also rapidly dropped in these corneas, and the mean ECD loss for T(Q)KO/*hCD46*/*hTBM* group was $61.7 \pm 5.2\%$ which was the highest. A recent study reported that the thrombomodulin gene is expressed 34 times more in Fuchs' corneal endothelial dystrophy [28]. Thrombomodulin expression may have led to iatrogenic Fuchs-like corneal endothelial dysfunction or apoptotic/necrotic cell loss. Although this study included a limited number of GE pigs, the findings suggest that GE pigs with *hTBM* may not be a suitable option for full-thickness corneal transplantation.

The study was limited to a limited number of GE pigs with a wide range of ages. The study faced constraints due to the high cost and limited availability of GE pigs. Using GE pigs solely for corneal studies was regarded as wasteful, which led to sharing GE pigs with other xenotransplantation teams for organs. Therefore, GE pigs of different ages were selected for the corneal study based

on the requirements of other xenotransplantation procedures. For example, to match the organ size for heart, kidney, and liver, a younger age of GE pigs was preferred, utilizing either T(Q)KO/hCD55/hCD39 or T(Q)KO/hCD46/hTBM pigs for organ and cornea. Whereas, older age of GE pigs was favored for islet xenotransplantation because a mature pancreas can provide a large number of islet cells. Aged QKO pigs were used for islet cell and cornea xenotransplantation. Despite the preliminary nature of the results due to these limitations, the study has still provided important insights for selecting optimal candidates in pig-to-NHP corneal xenotransplantation. Based on this data, we are currently conducting GE pig-to-NHP corneal transplantation (data not shown). Another limitation of this study was grouping TKO and QKO pigs together despite their heterogeneous gene profiles. In the context of other organ xenotransplantation, antigen recognition by NK and NKT cells is crucial, and a genetic deficiency in iGB3 may be theoretically advantageous for improving transplant outcomes [17]. However, there was a report that silencing the porcine iGb3s gene did not affect Gal α 3Gal levels or acute rejection in pig-to-NHP xenotransplantation [29]. Whereas, in corneal transplantation, NKT cells may have a role for tolerance [30]. Therefore, the effect of QKO versus TKO on rejection remains debated; that is, the effect of iGB3 KO is not definitive. However, this study focused on the biocompatible properties of the cornea, particularly the physiological functions of endothelial cells, rather than its immunological properties. Table S1 showed no significant differences in endothelial cell density (ECD), and Table S2 showed no difference in doubling time (DT) of endothelial cell proliferation between TKO and QKO. Accordingly, with regard to tests for the biological functions of endothelial cells, we included both TKO and QKO in the groups T(Q)KO/hCD55/hCD39 and T(Q)KO/hCD46/hTBM. The immunological behavioral differences between QKO and TKO should be further investigated.

Despite being of a preliminary nature with limited donor and recipient resources, this research is noteworthy for revealing the age relevance of GE pigs and the biocompatibility of pig corneal endothelial cells in T(Q)KO/hCD55/hCD39 and QKO for pig-to-NHP corneal xenotransplantation. The next step should involve conducting in vivo corneal xenotransplantation experiments with a sufficient sample size to evaluate the feasibility of using GE pigs in human clinical trials for corneal xenotransplantation for both biological and immunological aspects.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Table S1: Comparison of corneal endothelial cell density (ECD) and mean ECD loss in TKO/hCD46/hTBM or QKO/hCD46/hTBM pigs **Table S2:** Doubling time of corneal endothelial cells in TKO/hCD46/hTBM or QKO/hCD46/hTBM pigs