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Supplementary Materials for

Pathological processes in aqueous humor due to iris atrophy predispose to early corneal graft failure in humans and mice

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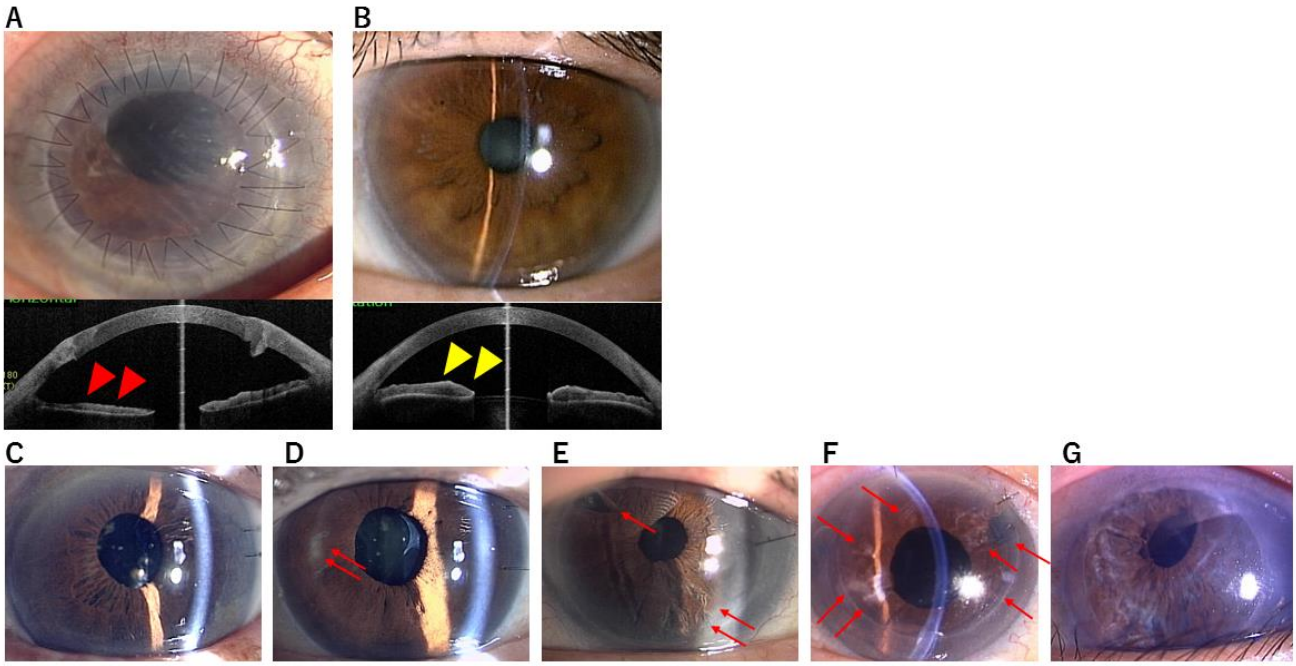

Fig. S1. A representative case with iris atrophy and definition of iris damage in human.

(**A**-**B**) A 78-year-old female with bullous keratopathy in the right eye. Patient had undergone corneal transplantation 5 times before and always ended in graft failure within a year. Anterior segment optical coherence tomography image showed severe iris atrophy in the bullous keratopathy eye (**A,** red arrow heads), whereas the left eye was normal (**B,** yellow arrow heads). Patient underwent a 6th penetrating keratoplasty in 2018. The preoperative protein/cytokine levels in AqH were extraordinarily elevated. Protein: 1.01 mg/mL (0.27 ± 0.04 mg/mL in healthy controls), IL-6: 209.6 pg/mL (6.4 ± 0.9 pg/mL), IFNγ: 387.6 pg/mL (54.5 ± 1.5 pg/mL), MCP-1: 1274 pg/mL (450 ± 25 pg/mL). (**C**-**G**) definition of iris atrophy, normal iris without any atrophy was defined as iris atrophy score (IAS) 0 (**C**), iris atrophy limited to one quadrant was defined as IAS1 (**D**), iris atrophy in two quadrant: IAS2 (**E**), iris atrophy in three quadrant: IAS3 (**F**) and whole iris atrophy: IAS4 (**G**). PHOTO CREDIT: Photographer: Takefumi Yamaguchi, MD Institution: Tokyo Dental College Ichikawa General Hospital

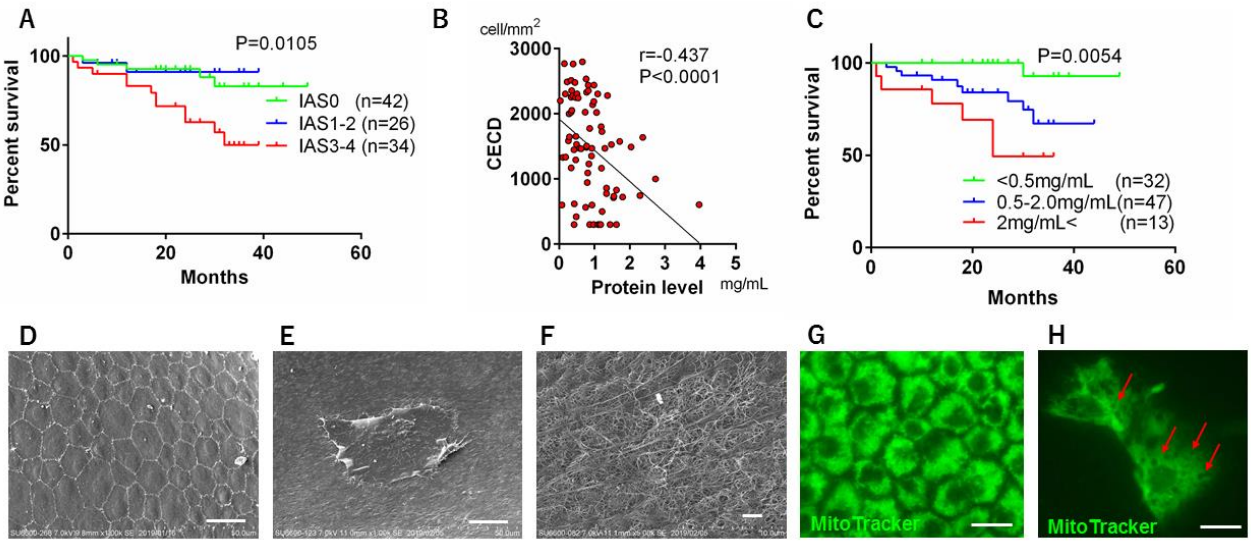

Fig. S2. Long-term clinical outcome of penetrating keratoplasty and scanning electron microscopy of bullous keratopathy eyes.

(A) Long-term graft survival stratified by iris atrophy severity after penetrating keratoplasty. The prognosis of eyes with severe iris damage score (IDS3-4) was significantly poor (*n* = 102, Log-rank Mantel-Cox test, P = 0.0105). (**B**) Correlation between preoperative protein levels in AqH and the CEnC density at 12 months after penetrating keratoplasty (Spearman's correlation analysis, r = -0.437, P<0.0001). Other time points are given in Table S2. (**C**) Graft survival was significantly shortened in eyes with high preoperative AqH protein levels compared to those with lower protein levels (Log-rank Mantel-Cox test, P<0.0001). (**C-F**) Scanning electron microscopy images of normal corneal endothelial cells (CEnCs, **C**) and bullous keratopathy (**D-E**). The corneal posterior surface revealed isolated dendritic-form CEnC (**D**) and degenerated collagen fibers of Descemet membrane (CEnCs basement membrane, **E**). Scale bar: D-E: 200 μm, F: 20 μm. MitoTracker staining representing mitochondrial mass. CEnCs of healthy CEnCs (**G**) and CEnCs of bullous keratopathy (**H**). In CEnCs of bullous keratopathy, we noted vesicles inside mitochondria (red arrows). Scale bar: 20 μm.

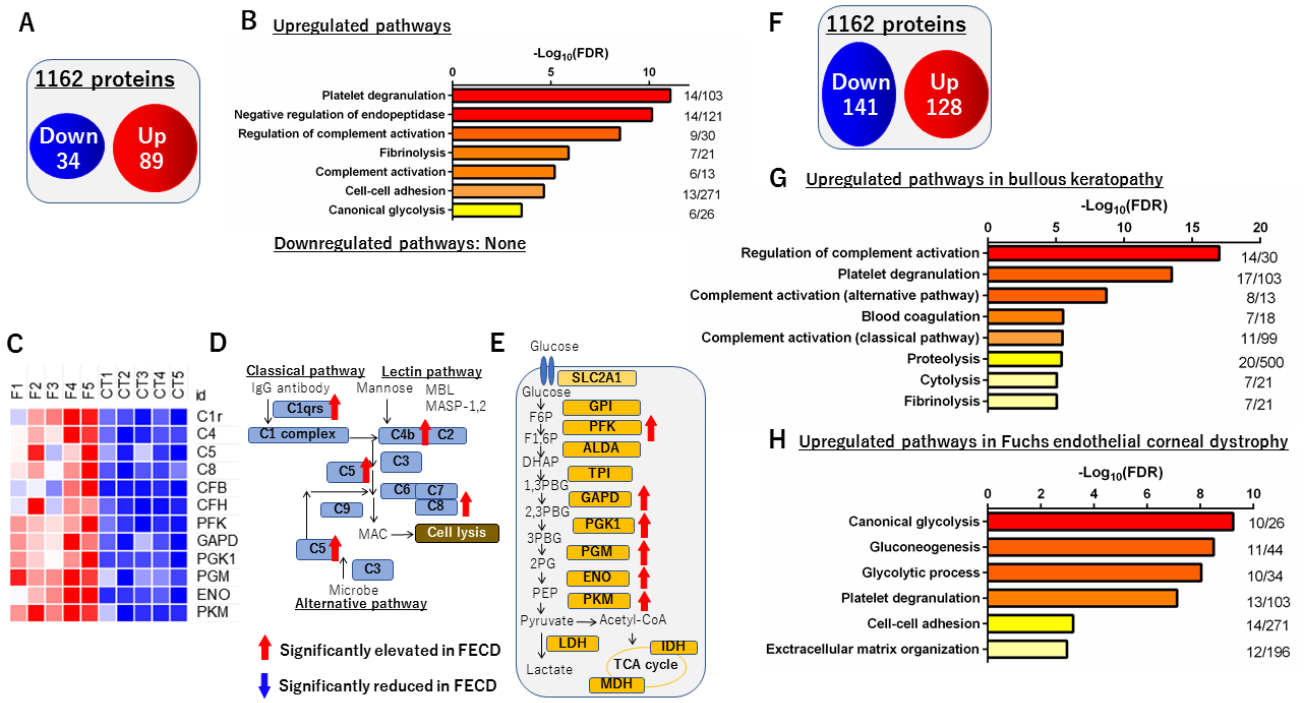

Fig. S3. Proteome analysis of aqueous humor in hereditary Fuchs' endothelial corneal dystrophy and bullous keratopathy.

(**A**) Venn diagram displaying the number of statistically significant identified altered proteins in eyes with hereditary Fuchs' endothelial corneal dystrophy (FECD, *n* = 5 eyes) compared to healthy controls (*n* = 5 eyes). (**B**) Canonical biological processes that were significantly upregulated in FECD were characterized using DAVID gene ontology analysis. We could not detect any significant biological processes associated with the list of downregulated proteins in FECD. (**C**) Heat map illustrating quantitative alterations of representative proteins (F: FECD, CT: healthy controls). (**D-E**) Proteins that are associated with complement activation (**D**) and glycolysis (**E**) were significantly increased in FECD as compared to healthy controls. (**F**) Venn diagram displaying the number of statistically significant identified altered proteins in bullous keratopathy eyes (*n* = 5 eyes) compared to hereditary Fuchs endothelial corneal dystrophy (FECD, *n* = 5 eyes). (**G**) Canonical biological processes that were significantly upregulated in bullous keratopathy compared to FECD were characterized using DAVID gene ontology analysis. (**H**) Canonical biological processes that were significantly reduced in bullous keratopathy compared to FECD.

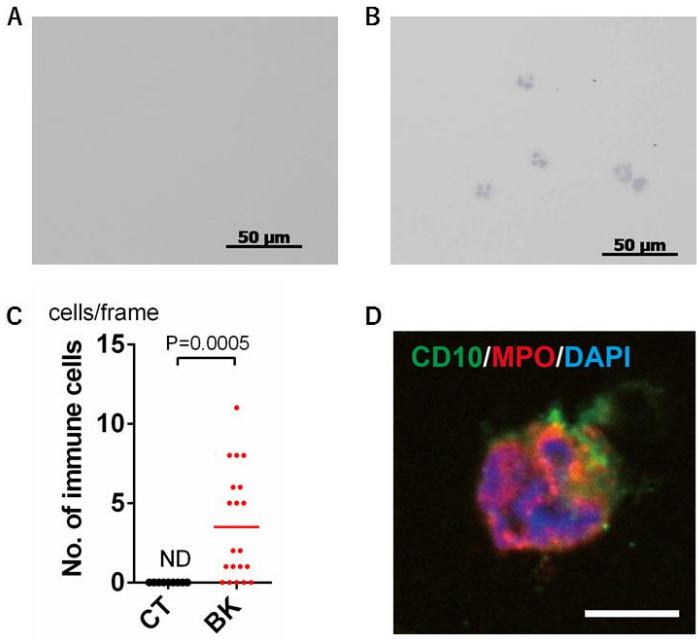

Fig. S4. Significantly increased immune cells in human bullous keratopathy AqH.

(**A**) Immune cells were not detected in AqH of healthy eyes (*n* = 4 eyes). (**B**) A significant number of immune cells were present in AqH of bullous keratopathy eye (*n* = 5 eyes). (**C**) Immune cell numbers were significantly greater in AqH of bullous keratopathy (BK), compared to healthy controls (CT, P=0.0005). Unpaired student T-test. (**D**) Immune cells were positive for CD10 and MPO. Scale bar: 10 µm.

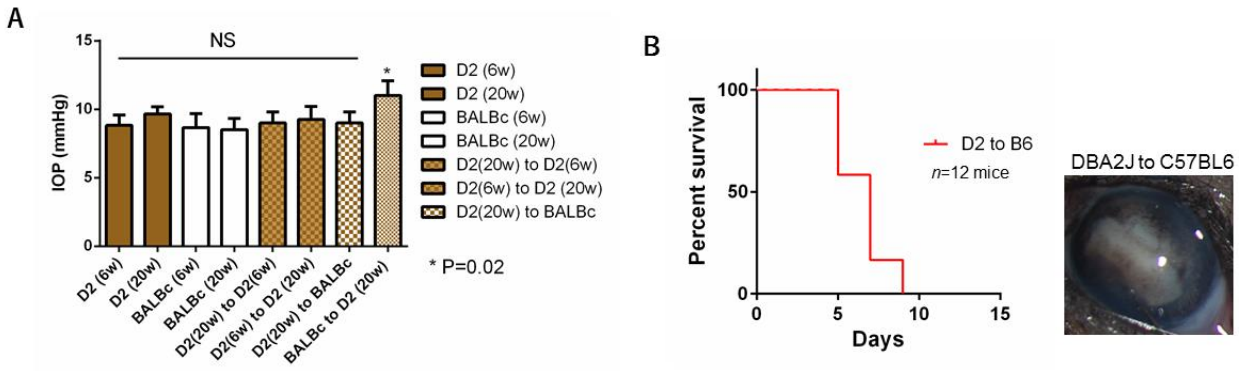

Fig. S5. Intraocular pressure in a steady state and after corneal transplantation.

(A) The DBA2J (D2) murine strain is a well-known model of pigmentary glaucoma that develops elevation of intraocular pressure at around 10-month of age. Since intraocular pressure increases can cause bias in the results, we evaluated the intraocular pressure in mice with and without surgical procedures. Only DBA2J mice after allogeneic transplantation (BALBc to DBA2J) exhibited significantly higher intraocular pressure relative to the other groups (*P=0.02). However, no significant differences were observed in intraocular pressure among the other groups. NS: not significant. Unpaired student T-test. **(B)** All the corneal graft from DBA2J into C57BL6 mice failed due to severe inflammation within 10 days after endothelial lamellar keratoplasty. PHOTO CREDIT: Photographer: Kazunari Higa, VDM Institution: Tokyo Dental College Ichikawa General Hospital

Table S1. Patient demographics in prospective long-term prognosis study undergoing corneal transplantation.

	Penetrating keratoplasty	Endothelial keratoplasty
No of patients	102 eyes of 102 patients	172 eyes of 172 patients
Male/Female	63 / 39	69 / 103
Patient age (years)	65.0 ± 17.3	73.8 ± 10.2
Follow up period (months)	23.9 ± 10.5	21.7 ± 10.1
Survival %	82 / 102 (80.3%)	149 / 172 (86.6%)
Axial length (mm)	24.94 ± 2.62	23.36 ± 1.69
Preop protein level in AqH (mg/mL)	0.88 ± 0.63	1.15 ± 0.78
Preop steroid use	30 / 102 (29.4%)	47 / 172 (27.3%)
Presence of glaucoma	25 / 102 (24.5%)	48 / 172 (27.9%)
Donor age (years)	64.6 ± 15.6	60.9 ± 11.6
Donor CECD (cells/mm^2)	2671 ± 317	2740 ± 246

CECD: corneal endothelial cell density

Table S2. Correlations between preoperative protein levels in aqueous humor and corneal endothelial cell density after corneal transplantation.

	Penetrating keratoplasty			Endothelial keratoplasty		
CECD	R	95% CI	P value	R	95% CI	P value
6 months	-0.263	-0.464 to -0.036	0.0202	-0.305	-0.456 to -0.136	0.0004
12 months	-0.437	-0.607 to -0.228	<0.0001	-0.408	-0.548 to -0.246	<0.0001
24 months	-0.515	-0.684 to -0.294	<0.0001	-0.492	-0.639 to -0.311	<0.0001
36 months	-0.487	-0.703 to -0.189	0.0019	-0.452	-0.659 to -0.311	0.0014

Spearman's correlations with preoperative protein levels and CECD at each timepoint

CI: confidence interval, CECD: corneal endothelial cell density

Table S3. Cox proportional hazard model using donor and recipient factors.

	Penetrating keratoplasty			Endothelial keratoplasty		
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
AqH protein level	**41.91**	**2.12 to 827.1**	**0.014**	**8.62**	**1.88 to 39.6**	**0.006**
Patient age	0.94	0.87 to 1.01	0.100	0.97	0.93 to 1.02	0.194
Preop steroid use	0.29	0.05 to 1.57	0.153	1.51	0.54 to 4.18	0.432
Presence of glaucoma	3.05	0.67 to 14.0	0.151	2.52	0.94 to 6.72	0.064
Donor age	1.02	0.97 to 1.07	0.494	1.01	0.97 to 1.05	0.697
Donor CECD	1.00	0.99 to 1.00	0.844	0.99	0.99 to 1.00	0.907

*† Presence of glaucoma, protein levels and steroid use were dichotomized as categorical variables for multivariate regression analysis as follows: presence of glaucoma: absence = 0, presence = 1, AqH protein level: <1.0mg/mL=0, 1.0mg/mL≤ =1, steroid use: absence = 0, presence = 1, HR: hazard ratio, CI: confidence interval, CECD: corneal endothelial cell density, VIF: variance inflation factor, 1.14 to 1.16

Clinical factors influencing graft survival after corneal transplantation

The results can be influenced by other clinical factors, such as postoperative medication, presence of glaucoma, and other factors, which could have caused bias. For example, a history of glaucoma has been reported to be one of risk factor for poor prognosis (*9, 11*). However, the prognosis was relatively fair in glaucoma patients with low preoperative protein AqH levels, whereas it was very poor in glaucoma patients with high protein levels in AqH (Fig. 1F, no grafts survived in eyes with AqH protein level≥2.0mg/mL). To evaluate the association between graft survival, AqH protein level and these clinical factors, we conducted Cox proportional hazard model which revealed that preoperative AqH protein level was significantly associated with poor graft prognosis (table S3, HR = 41.91, P = 0.014 in penetrating keratoplasty, HR = 8.62, P = 0.006 in endothelial keratoplasty).

Table S4. Demographics of patients for electron microscopy/ proteome/ transcriptome analysis.

Electron microscopy	Healthy controls	Bullous keratopathy	Corneal diseases	P value
No. of patients	5	6	7	
Male / Female	4 / 1	4 / 2	5/2	1.00
Age (years)	56.6 ± 14.3	77.4 ± 7.3	66.5 ± 15.8	0.08
CECD	2512 ± 143	NA	NA	

Proteome	Healthy controls	Bullous keratopathy	FECD	P value
No. of patients	5	5	5	
Male / Female	2 / 3	3 / 2	2/3	1.00
Age (years)	77.6 ± 11.4	59.8 ± 21.4	65.1 ± 15.4	0.08
Axial length (mm)	23.6 ± 1.4	24.4 ± 3.2	23.9 ± 2.1	0.32
CCT (μm)	512 ± 23	764 ± 91	638 ± 105	0.0013
Iris atrophy score	0 ± 0	2.6 ± 0.4	0 ± 0	0.001

Transcriptome	Healthy controls	Bullous keratopathy	P value
No. of patients	4	7	
Male / Female	2 / 2	3 /4	1.00
Age (years)	62.8 ± 12.0	73.4 ± 6.7	0.09
CCT (μm)	NA	648 ± 52	
Iris atrophy score	NA	1.6 ± 0.8	<0.0001
CECD (cells/mm^2)	2726 ± 181	380 ± 172	<0.0001

FECD: Fuchs endothelial corneal dystrophy, CCT: central corneal thickness, NA: not available because corneas of healthy controls are from US corneal donors, CECD: corneal endothelial cell density

Table S5. Demographics of patients for aqueous humor cytokine/8-OHdG analysis.

Cytokine	Healthy controls	Bullous keratopathy	P value
No. of patients	51	194	
Male / Female	22 / 29	87 / 107	0.88
Age (years)	75.2 ± 9.7	72.1 ± 11.1	0.07
Axial length (mm)	24.0 ± 1.8	23.7 ± 2.1	0.38
CCT (μm)	514 ± 52	743 ± 134	<0.001
8-OHdG			
No. of patients	27	33	
Male / Female	9 / 18	12 / 21	1.00
Age (years)	79.7 ± 7.9	71.6 ± 12.5	0.003
Axial length (mm)	24.5 ± 2.8	23.2 ± 1.5	0.03
CCT (μm)	526 ± 12	744 ± 129	<0.001

CCT: central corneal thickness