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Janus kinase inhibitors in the treatment of refractory cicatrizing conjunctivitis in pemphigoid

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ABSTRACT

Objective: To evaluate the efficacy of Janus kinase inhibitor (JAKi) therapy in managing cicatrizing conjunctivitis associated with ocular cicatricial pemphigoid (OCP) and mucous membrane pemphigoid with ocular involvement (ocMMP).

Methods: Retrospective chart review of patients with cicatrizing conjunctivitis secondary to OCP or ocMMP who underwent treatment with JAKi at a tertiary academic medical center from August 2015 to November 2024 for minimum follow-up of six months. Collected data included demographics, Foster stage of cicatrization, and treatment course.

Results: Thirty-two patients met inclusion criteria: 23 (71.9 %) with OCP and 9 (28.1 %) with ocMMP. 96.9 % of patients demonstrated clinical improvement within twelve months of treatment initiation. Best response achieved were as follows: 1 (3.1 %) no response, 17 (53.1 %) partial response, 14 (43.8 %) complete remission, and 12 (37.5 %) steroid-free complete remission. The mean time to partial response, complete remission, steroid-free complete remission was 3.1 ± 1.8 (range, 0.9–8.3), 7.8 ± 3.3 months (range, 2.3–14.7 months), and 10.3 ± 7.4 months, (range, 2.3–31.4 months), respectively. Relapse in disease activity occurred in 8/32 (25.0 %) of patients. Side effects occurred in 8/32 (25.0 %) of patients. Four patients (12.5 %) discontinued therapy due to severe adverse events, including transient ischemic attack, pulmonary embolism, pyelonephritis, and cholecystitis. There was a significant association between lower Foster cicatrization stages and achieving remission ($U = 630.0$, $p = 0.0036$), with a rank-biserial correlation of 0.72.

Conclusions: JAK inhibitor therapy demonstrates efficacy in the management of recalcitrant cicatrizing conjunctivitis associated with pemphigoid. These findings highlight JAK inhibitors as a promising therapeutic option for refractory cases.

1. Introduction

Mucous Membrane pemphigoid (MMP) is a systemic autoimmune bullous disease characterized by subepithelial blistering primarily involving the mucous membranes and skin [1,2]. Ocular cicatricial pemphigoid (OCP) is a subset of MMP involving the ocular surface [3]. The disease is characterized by conjunctival inflammation resulting in symptoms of pain, photophobia, tearing, and blurred vision [3]. The degree of cicatrization secondary to inflammation is characterized by subepithelial fibrosis, fornix foreshortening, symblepharon formation, and finally, limbal stem cell deficiency and corneal keratinization leading to irreversible blindness [4].

The pathogenesis of MMP is characterized by a complex, dysregulated autoimmune response targeting antigens within the basement membrane zone of various mucosal surfaces [5,6]. While often conceptualized as an antibody-mediated type 2 hypersensitivity reaction, the underlying immunopathogenic mechanisms are complex and not fully understood [5,6] Current literature suggests the cytoplasmic domain of the β4 peptide of α6β4 integrin, a transmembrane anchoring protein, as a target of this aberrant immune reaction [5]. Other antibody targets are integral features of the basement membrane zone, such as BP180 and BP230 [2]. Additionally, the human leukocyte antigen (HLA) DQB1*0301 allele has been identified in higher frequencies in patients with OCP with a prevalence of up to 75 % [7,8]. While these molecular

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identifiers have been found to have a relationship with the disease, their presence is not required for diagnosis and does not provide definite prognostic value [6,9].

JAK proteins, a non-receptor tyrosine protein kinase, are essential in the differentiation and proliferation of T-helper (TH) 17 cells, which secrete cytokines including IL-17 and IL-22 [10,11]. The interaction of cytokines with the JAK-STAT pathway is believed to be involved in pemphigoid diseases, including MMP [10]. MMP is associated with an increased localization of TH17 lymphocytes, especially in the conjunctiva, and the overexpression of IL-6, IL-12, and IL-17 [10,12]. STAT3 antibody binding within the conjunctiva leads to the secretion of proinflammatory cytokines and complement proteins [13]. These cytokines bind receptors that activate various JAK-STAT pathways which upregulate gene transcription in the nucleus of cells leading to increased levels of inflammation [13].

JAK inhibitors (JAKi) function by binding to the kinase domain of JAK proteins preventing their activation by cytokines [13]. This inhibits the further signaling that occurs through STAT phosphorylation and averts the activation of pro-inflammatory gene expression [13]. Baricitinib and tofacitinib are two JAKi that have been approved for use in autoimmune conditions including rheumatoid arthritis, alopecia areata, ulcerative colitis, and ankylosing spondylitis [13]. Baricitinib is primarily a JAK1 and JAK2 inhibitor, while tofacitinib is primarily a JAK 1 and JAK 3 inhibitor [13]. Limited case reports have described the efficacy of JAK inhibition in the treatment of ocular inflammatory conditions such as scleritis and uveitis [14,15]. .We herein describe the successful use of JAKi in patients with cicatrizing conjunctivitis associated with MMP and OCP.

2. Materials/methods

This study was conducted at the University of Texas Southwestern Medical Center and received approval from the university's Institutional Review Board (IRB). All aspects of patient data handling adhered to the Health Insurance Portability and Accountability Act (HIPAA) and the tenets of the Declaration of Helsinki.

A retrospective chart review was performed for patients seen in the University of Texas Southwestern Ophthalmology and Dermatology clinics between August 2015 and November 2024. Patients included in the study were diagnosed with cicatrizing conjunctivitis secondary to ocular cicatricial pemphigoid (OCP) or mucous membrane pemphigoid with ocular involvement (ocMMP) undergoing treatment with a Janus kinase (JAK) inhibitor due to active ocular disease who had at least six months follow-up.

The diagnosis of OCP or ocMMP was confirmed through clinical evaluation by a board-certified uveitis fellowship-trained ophthalmologist (JHC) and a board-certified dermatologist (AD), both specializing in autoimmune blistering diseases. In cases with negative conjunctival or skin biopsies, diagnosis was based primarily on characteristic clinical features [16], including progressive conjunctival cicatrization, serologic testing for BP180/BP230 antibodies, HLADQB1*0301 testing, and exclusion of other etiologies of cicatrizing conjunctivitis such as Stevens-Johnson syndrome, atopic keratoconjunctivitis, and ocular rosacea.

Data collection included patient demographics such as age, sex, and race, medical and ocular history, clinical findings, conjunctival and buccal biopsy results for direct immunofluorescence, genetic markers (HLA DQB1*0301), serum markers including BP180 and BP230 antibodies, indirect immunofluorescence using 1M NaCl-split skin as the substrate, Foster cicatrization staging, and previous and current treatment regimens. Use of concomitant steroids, treatment response, and adverse events were also recorded.

Initiation of JAKi therapy was based on failure or intolerance of prior immunosuppressive treatments and evidence of ongoing active disease (inflammation) on clinical examination. The choice of JAKi, either tofacitinib or baricitinib, was determined by medication availability, insurance approval, and/or access to drug-sponsored patient assistance programs. There was no washout period for previous therapy. Initial dosing consisted of tofacitinib 5 mg twice daily or baricitinib 2 mg once daily. Typically, all other immunosuppressive medications, except intravenous immunoglobulin (IVIG) if applicable, were discontinued. Dosing was subsequently titrated based on clinical response to a maximum of tofacitinib 10 mg twice daily or baricitinib 4 mg once daily. If conjunctival or extra-ocular disease persisted, an antimetabolite was then added. Once remission was achieved, topical and oral steroids were tapered as tolerated.

Patients underwent regular clinical examinations at 1 month, 3 months, 6 months, and then at regular 3–4-month intervals. Disease cicatrization staging was performed using the Foster grading scale for cicatrization [4]. Disease activity was determined by degree of conjunctival inflammation as previously described [17]. Slit-lamp photography was performed at baseline and periodically throughout the treatment period to document disease progression and response to therapy. At intervals of 6-, 12-, 18-, and 24-months following initiation of JAKi therapy, patients were categorized into four response groups based on ocular clinical disease activity. Response categories were defined as follows: no response (no improvement in inflammation), partial response (interval decrease in inflammation), complete remission (no active ocular disease-related inflammation with concomitant steroid use), and steroid-free complete remission (no ocular disease activity in the absence of any corticosteroid use) [18]. Extra-ocular disease response to treatment was not recorded.

Statistical analyses were performed using R (R Core Team, Vienna, Austria). Descriptive statistics summarized baseline characteristics, with continuous variables reported as means ± standard deviations or medians with interquartile ranges, and categorical variables as counts and percentages. Comparative analyses included the Mann-Whitney *U* test for nonparametric data and Fisher's exact test for categorical variables. A rank-biserial correlation coefficient quantified associations for significant findings, with a two-sided p-value <0.05 considered statistically significant. To explore potential correlations between treatment response and biomarker or histopathologic findings, we analyzed categorical variables including BP180, BP230, HLA DQB1*0301 status, and conjunctival and buccal biopsy results. Given the small sample size, Fisher's exact test was used for all comparisons.

3. Results

3.1. Study population

A total of 115 patients with OCP and ocMMP were identified. Of these, 37 patients underwent treatment with JAKi and met the initial inclusion criteria. Four patients were excluded due to lack of follow-up, and one patient was excluded due to absence of active ocular inflammation at the time of drug initiation, resulting in a final cohort of 32 patients. Of note, one patient in our cohort was previously included in an individual case report published in 2022 [19].

Baseline demographic data is shown in Table 1. Nineteen (59.4 %) patients were female. The mean age of disease onset was 55.6 ± 12.1 years-old (range, 33.8–81.0 years). The mean age of diagnosis was 62.0 ± 10.9 years-old (range, 37.3–82.6 years). A total of thirty-one (96.9 %) patients were Caucasian and one patient was Hispanic. A total of 23 patients (71.9 %) had ocular cicatricial pemphigoid (OCP), and 9 (28.1 %) had ocular mucous membrane pemphigoid (ocMMP). All patients underwent mucosal biopsy as part of the workup for MMP/OCP; 25 (78.1 %) were biopsy positive on direct immunofluorescence. There were 18/25 (69.2 %) positive conjunctival biopsies and 12/23 (52.2 %) positive buccal biopsies. HLA-DQB1*0301 allele was detected in 13/27 (48.1 %). BP230 and BP180 antibodies were detected in 3/14 (21.4 %) patients. Prior to initiation of JAKi, our cohort had failed the following therapies: tacrolimus (1, 3.1 %), azathioprine (3, 9.4 %), methotrexate (3, 9.4 %), mycophenolate (23, 71.9 %), rituximab (16, 50.0 %),

Table 1
Patient demographics.

Characteristic	n (%)	Mean ± SD
Gender		
Female	19 (59.4 %)	
Male	13 (40.6 %)	
Race		
Non-Hispanic White	31 (96.9 %)	
Hispanic White	01 (3.1 %)	
Diagnosis		
OCP[a]	23 (71.9 %)	
ocMMP[b]	09 (28.1 %)	
Timing		
Age of Onset		55.6 ± 12.1
Age of Diagnosis		62.0 ± 10.9
Duration of Disease at JAKi initiation (y)		9.4 ± 6.6
MMP Extraocular Manifestations	12 (37.5 %)	
Oral	10 (31.3 %)	
Laryngeal, Tracheal, Esophageal	04 (12.5 %)	
Anogenital	04 (12.5 %)	
Cutaneous	02 (6.3 %)	
Foster Cicatrization Stage (eye)		
1	08 (12.5 %)	
2	14 (21.9 %)	
3	26 (40.6 %)	
4	16 (25.0 %)	
Biopsy		
Any Biopsy	32 (100 %)	
Positive	25 (78.1 %)	
Negative	7 (21.9 %)	
Conjunctival Biopsy	26 (81.2 %)	
Positive	18 (69.2 %)	
Negative	8 (32.0 %)	
Buccal Biopsy	23 (71.9 %)	
Positive	12 (52.2 %)	
Negative	11 (47.8 %)	
Antibodies		
BP230		
Positive	03 (21.4 %)	
Negative	11 (78.6 %)	
BP180		
Positive	03 (21.4 %)	
Negative	11 (78.6 %)	
HLA-DQB1*0301 Status		
Positive	13 (48.1 %)	
Negative	14 (51.9 %)	
Extra-ocular Mucosa and Cutaneous Involvement		
Oral	12 (37.5 %)	
Nasal	03 (9.4 %)	
Pharyngeal	01 (3.1 %)	
Esophageal	02 (6.3 %)	
Anogenital	04 (12.5 %)	
Cutaneous	02 (6.3 %)	
Previously Failed Therapies		
Tacrolimus	01 (3.1 %)	
Azathioprine	03 (9.4 %)	
Methotrexate	03 (9.4 %)	
Mycophenolate mofetil	23 (71.9 %)	
Rituximab	14 (43.8 %)	
Bortezomib	02 (6.2 %)	
Intravenous Immunoglobulin	08 (25.0 %)	
Cyclophosphamide	07 (21.9 %)	

[a] OCP: ocular cicatricial pemphigoid (isolated ocular disease).
[b] ocMMP: ocular mucous membrane pemphigoid (ocular disease with additional extraocular disease).

bortezomib (2, 6.3 %), IVIG (8, 25.0 %), and cyclophosphamide (7, 21.9 %). For the patients with ocMMP, extra-ocular manifestations included oral (12, 37.5 %), nasal (3, 9.4 %), pharyngeal (1, 3.1 %), esophageal (2, 6.3 %), anogenital (4, 12.5 %), and cutaneous (2, 6.3 %) involvement.

A total of 64 eyes from 32 patients with OCP/MMP were assessed using the Foster Classification System: 8 (12.5 %) were Stage 1, 14 (21.9 %) were Stage 2, 26 (40.6 %) were Stage 3, and 16 (25.0 %) Stage 4 (Table 1). Visual acuity (logMAR) at the start of JAKi therapy was 0.34 ± 0.36 (range, 0.0–1.30) in the better seeing eye and 0.82 ± 0.76 (range, 0.0–2.8) in the worse seeing eye. The medium duration from diagnosis of MMP or ocMMP to the start of JAKi treatment was 9.3 ± 6.6 years (range, 1.9–23.6 years; Table 2)

3.2. Treatment outcomes

In our study cohort, the choice of JAKi, either tofacitinib or baricitinib, was determined by route of medication access: 7 (21.9 %) through insurance, 18 (56.3 %) through Patient Assistance Programs, and 7 (21.9 %) through an international self-pay pharmacy. Twenty-two patients (68.8 %) initiated therapy with baricitinib (2–4 mg/day), while 10 (31.3 %) initiated tofacitinib (10–20 mg/day). Four patients subsequently transitioned from tofacitinib to baricitinib: three due to challenges related to medication access, including insurance approval and eligibility for manufacturer-sponsored patient assistance programs, and one due to inadequate therapeutic response on tofacitinib. One patient transitioned from baricitinib to tofacitinib, then back to baricitinib due to challenges related to medication access (Fig. 2). The medium duration of JAKi treatment and time of follow-up were 18.5 ± 12.5 months (range, 6.7–57.7 months), and 18.9 ± 12.4 months (range, 6.7–57.7 months), respectively (Table 2).

The clinical course of our cohort is summarized in Fig. 2. In our study, 96.9 % of patients showed a positive response to medication within 12 months. The proportion of patients achieving complete remission or better by 6, 12, 18, and 24 months were 4/32 (12.5 %), 13/32 (40.6 %), 14/32 (43.8 %), and 14/32 (43.8 %), respectively (Fig. 2). The proportion of patients achieving steroid-free complete remission by 6, 12, 18, and 24 months were 2/32 (6.3 %), 10/32 (31.3 %), 11/32 (34.4 %), and 11/32 (34.4 %), respectively (Fig. 2). There was no statistically significant difference in rates of achieving disease remission based on biopsy positivity (66.7 % vs. 14.3 %; OR 4.0, $p = 0.212$). A series of conjunctival photographs demonstrating selected clinical responses to JAKi are demonstrated in Figure 1.

The mean time to partial response, complete remission, and steroid-free complete remission were 3.1 ± 1.8 (range, 0.9–8.3), 7.8 ± 3.3 months (range, 2.3–14.7 months), and 10.3 ± 7.4 months, (range, 2.3–31.4 months), respectively (Table 2).

During treatment with JAKi, 10 patients were on concurrent therapy with immunomodulators: methotrexate (2, 6.3 %), mycophenolate (6, 18.9 %), and IVIG (5, 15.6 %), and two (6.3%) patients were also on concomitant systemic corticosteroids (prednisone)

A total of 8 patients (25.0 %) had a relapse in disease activity. Of those 14 patients that achieved any degree of complete remission, 4 (28.6 %) had a relapse in disease activity. The mean time to relapse following best response to treatment was 12.8 ± 7.5 months, (range 5.2–24.7 months) (Table 2). Although full remission for these patients was not regained, inflammation remained partially controlled.

There was a statistically significant difference in Foster stages between those that achieved partial response (n = 40) vs those that achieved remission (n = 22) (U = 630.0, $p = 0.0036$). The rank-biserial correlation was calculated as 0.72; eyes with lower Foster stages (lower pre-existing ocular burden) were associated with a higher

Table 2
Time to achieve clinical milestones in patients treated with JAK inhibitors.

	n	Mean ± STD (mos)	Range (mos)	Median (mos)
Duration of JAK inhibitor Treatment	32	18.5 ± 12.5	6.7–57.7	15.2
Total Follow-Up Time	32	18.9 ± 12.4	6.7–57.7	15.6
Time to Partial Response	31	3.1 ± 1.8	0.9–8.3	03.1
Time to Complete Remission	14	7.8 ± 3.3	2.3–14.7	08.1
Time to Steroid-Free Complete Remission	12	10.3 ± 7.4	2.3–31.4	08.7
Time to Relapse	8	12.8 ± 7.5	5.2–24.7	10.2

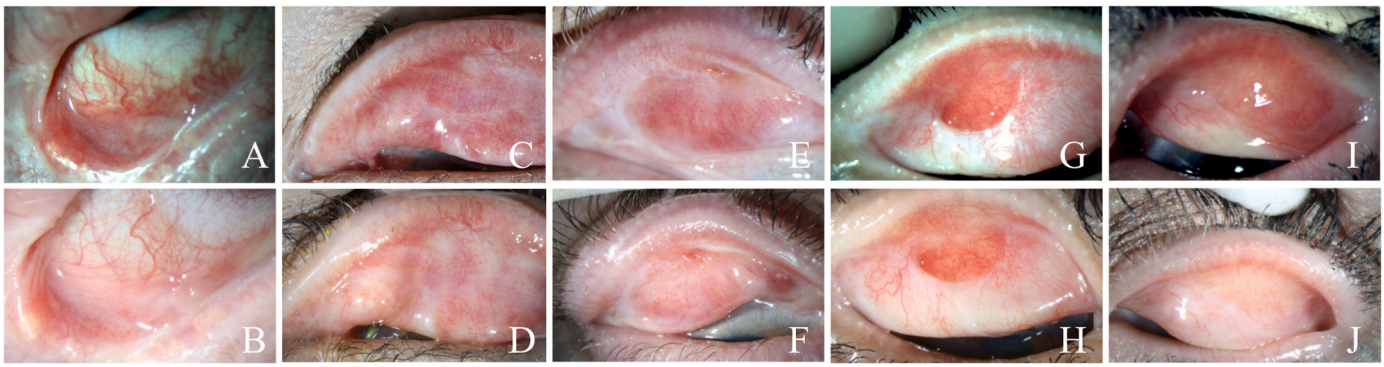

Fig. 1. Clinical response to Janus kinase inhibitor (JAKi) inhibitor therapy in patients with Ocular Pemphigoid
(A-B) Left upper eyelid of a 68 y/o monocular female with Mucous membrane pemphigoid (MMP), Foster Stage 4. She previously failed azathioprine, mycophenolate, rituximab, and IVIG (Figure A). She demonstrated full response of the left upper eyelid (Figure B, 15 months post-initiation), but partial response in the right eye on baricitinib 4 mg daily. (C–D) Right lower eyelid of an 84 y/o female with ocular cicatricial pemphigoid (OCP), Foster Stage 3 cicatrization. She previously failed azathioprine and infliximab. Figure C demonstrated continued inflammation on rituximab 1g Q6months + IVIG 1g Qmonth. Patient achieved steroid-free complete remission on baricitinib 2 mg daily (Figure D, 4 months post initiation). (E–F) Right upper eyelid of a 46 y/o male with OCP, Foster Stage 3 cicatrization, failing mycophenolate mofetil (MMF) 1500 mg PO BID (Figure E). He achieved partial response on baricitinib 4 mg daily (Figure F, 17 months post-initiation). (G–H) Left upper eyelid of a 71 y/o monocular female with MMP, Foster Stage 2 cicatrization. She previously failed tacrolimus and mycophenolate mofetil 1500 mg PO BID (Figure G). There was a partial response on baricitinib 2 mg daily (Figure H, 4 months post-initiation), which was discontinued after failing a cardiac nuclear medicine stress test. (I–J) Right upper eyelid of a 47 y/o monocular female with MMP, Foster Stage 4 who had previously failed azathioprine, infliximab, rituximab, IVIG, and cyclophosphamide (Fig. I). Patient demonstrated full response of the right eye (Figure J, 15 months post-intitiation) but partial response of the other eye on baricitinib 4 mg daily + MMF 1500 mg PO BID.

likelihood of achieving remission when compared to eyes with higher Foster stages. No statistically significant associations were observed between treatment response and BP180, BP230, HLA status, or conjunctival and buccal biopsy findings (all $p > 0.05$, Fisher's exact test).

3.2.1. Side effects and adverse events

Side effects were observed in 8 patients (25.0 %) in the cohort, including gastrointestinal symptoms (3 patients, 9.4 %), facial acneiform eruptions (3 patients, 9.4 %), joint pain (1 patient, 3.1 %), abdominal pain (1 patient, 3.1 %), and weight gain (1 patient, 3.1 %). One patient with facial acneiform eruptions temporarily discontinued therapy for four months but successfully resumed treatment; all 3 patients' acinetiform disease was managed successfully with typical topical therapy or doxycycline. Another patient treated with baricitinib interrupted therapy due to elevated serum creatinine (from baseline Cr 1.11/eGFR 55 to peak Cr 1.95/eGFR 28), which resolved after three months. However, challenges in medication access extended the treatment gap to eight months.

There were 4 (12.5 %) significant adverse events: sepsis secondary to cholecystitis (1, 3.1 %), pyelonephritis (1, 3.2 %), transient ischemic attack (TIA) (1, 3.1 %), and pulmonary embolism (PE) (1, 3.1 %). All 4 patients have discontinued JAKi therapy.

4. Discussion

Currently, systemic corticosteroids remain the only FDA-approved treatment for ocular pemphigoid. However, in clinical practice, a broad range of immunomodulatory therapies, including methotrexate, mycophenolate mofetil, rituximab, intravenous immunoglobulin (IVIG), and cyclophosphamide, have been employed to mitigate disease progression [20]. Stepwise treatment algorithms, initially proposed by Chan et al., have been internationally adopted, leveraging the Foster staging system to stratify therapies based on disease severity [1,21,22]. Despite these efforts, current therapies achieve remission rates averaging 50 %, with sustained remission beyond six months being notably uncommon [23–25].

Baricitinib and tofacitinib are both categorized as first-generation Janus kinase (JAK) inhibitors and exhibit a comparable spectrum of cytokine blockade [26]. Baricitinib preferentially inhibits JAK1 and JAK2 [27], whereas tofacitinib is selective for JAK1 and JAK3 [28]. Emerging evidence from recent case reports have begun to highlight the potential efficacy of JAKi, such as baricitinib and tofacitinib, in the management of treatment-refractory mucous membrane pemphigoid, with a few case reports for ocular response in this disease [29,30]. However, data is limited and evidence for the role of these medications is still growing. Sarny et al. (2018) [29] documented the first successful application of baricitinib, combined with methotrexate, in achieving remission in an OCP patient previously unresponsive to mycophenolate mofetil, IVIG, and cyclophosphamide. Clinical follow-up demonstrated significant improvement, including reduced conjunctival hyperemia and corneal neovascularization. Similarly, James et al. (2021) [30] described two patients with OCP achieving sustained remission following tofacitinib initiation, even after prior failures with multiple immunosuppressive agents. Both patients reported no significant adverse events, and complete remission occurred as early as one-month post-treatment, with sustained effects lasting over a year.

In this study, 33/34 (93.8 %) of patients exhibited clinical improvement following six months of JAKi therapy, with 14/32 (37.5 %) achieving complete remission or better after twelve months. The small sample size, retrospective design, and absence of a control group in this study may introduce bias, particularly as the initial response rates exceeded 90 %, and many patients subsequently reverted back to a "partial response" status. However, notably, this cohort included a highly refractory population, with 17 patients (53 %) having previously failed rituximab ± IVIG and/or cyclophosphamide [31,32], considered the current gold standard treatments reserved for the most recalcitrant cases of pemphigoid [20,33]. In our cohort, the best response included 14 patients (43.8 %) achieving complete remission, and 12 patients (37.5 %) achieving steroid free complete remission, with a mean time to complete remission of 7.8 months. It is important to note that of the 14 patients who previously failed rituxan, there were 5 (36%) patients who converted directly a JAK inhibitor within 6 mos of their last rituxan dose. Of these patients, 2 (40%) achieved steroid free complete remission at 5 and 8 months and have maintained this status for 30 and 16 months to date, respectively. The other three (60%) patients never achieved better than partial remission at any time course. This indicates that peri-initiation exposure to rituximab did not influence ultimate treatment outcome to JAK therapy.

These results underscore the potential efficacy of JAKi in a heavily

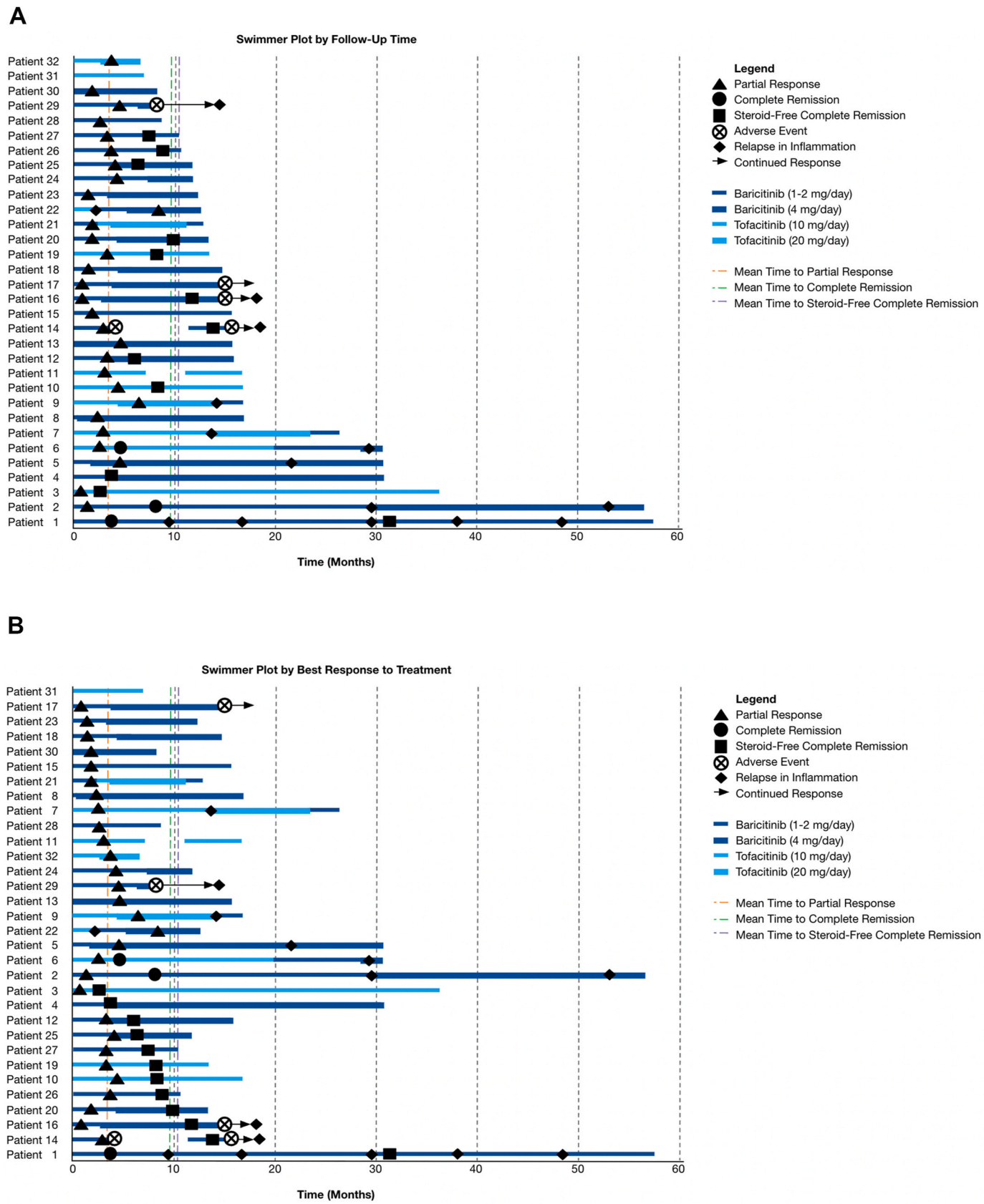

Fig. 2. Swimmers Plot of Clinical Course on JAK Inhibitor Therapy for Pemphigoid
Swimmers plot demonstrating clinical response ordered by follow-up time to initiation of JAK inhibitors (A) and best response to treatment with JAK Inhibitors at any point in treatment course (B). Complete remission was defined as no active disease-related activity but allows for any concomitant steroid use. Steroid-free complete remission was defined as no disease activity in the absence of any corticosteroid use.

pretreated, refractory population, where overall response rates are likely underestimated compared to a treatment-naïve cohort. Additional patients in our cohort had contraindications to rituximab ± IVIG due to comorbidities. Furthermore, social determinants of health posed barriers to access for additional patients (3, 9.4 %) due lack insurance coverage, inability to secure reliable transportation for infusions, and suboptimal living conditions for which home infusion services were unable to provide care. In this context, JAKi as a once or twice daily home-administered oral medication served as an important therapeutic alternative. Additional prospective randomized controlled trials are needed to further understand the role of JAKi in the current existing stepladder treatment algorithm for pemphigoid.

This study was neither designed nor powered to evaluate the superiority of one JAKi over another based on their selectivity profiles. Access to these medications within the cohort was influenced by various factors, including insurance approval, and alternative sourcing including patient assistance programs (PAPs) and international pharmacies. This resulted in a cohort skewed towards patients aged 65 years and older. Further randomized controlled studies will be necessary to further distinguish targeted inhibition of which JAKi(s) is most critical for disease control in ocular pemphigoid.

Notably, a partial response to rituximab and/or cyclophosphamide was an indication for a therapeutic trial of a JAKi therapy. Distinguishing between patients who achieved partial remission on JAKi and those who had "failed" rituximab and/or cyclophosphamide was challenging, as partial remission with these prior therapies frequently prompted the transition to JAKi treatment. However, review of slit lamp photography suggests the response to JAKi was, at minimum, non-inferior. All patients achieving partial response to JAKi were given the option to either continue JAKi therapy or restart previous regimens. All patients in this subgroup chose to remain on JAKi therapy, citing comparable efficacy to treatments, relative intensity associated with rituximab and IVIG infusions and/or risks of chronic cyclophosphamide therapy, accessibility challenges, and limited alternative therapeutic options.

Patient preference was a significant factor in treatment decisions, and no patients in our cohort opted to discontinue JAKi therapy unless a serious adverse events continuation contraindicated. Of note, two patients who discontinued JAKi due to a serious adverse event experienced relapse within 6 months of treatment discontinuation despite transition to mycophenolate, while a third patient who discontinued for less than 2 months experienced relapse within 5 months of treatment discontinuation.

While JAKi showed promise in managing ocular disease in refractory pemphigoid, adverse events occurred in four patients (12.5 %), including PE, transient ischemic attack (TIA), cholecystitis with sepsis, and acute pyelonephritis (in the setting of chronic bacterial urinary colonization and recurrent urinary tract infections (UTI)). Although no patients had a history of thromboembolic events at treatment initiation, the advanced age and comorbidities within this cohort may have predisposed them to higher complication rates. These findings emphasize the importance of careful patient selection and counseling before initiating JAKi therapy, particularly in older populations or those with preexisting comorbidities [34].

Two patients (6.3 %) in our study temporarily discontinued JAK inhibitor therapy due to adverse events, including acneiform eruptions and elevated serum creatinine. One patient achieved complete remission prior to discontinuation, while the other remained in partial remission after reinitiating treatment. Studies suggest that treatment interruptions or dose reductions of JAK inhibitors may result in diminished efficacy upon reinitiation, potentially due to JAK2 activation loop phosphorylation [35], JAK1-dependent transphosphorylation [36], or transient disease exacerbation following withdrawal [37]. Nonetheless, these studies suggest that efficacy can often be regained over time, typically requiring several weeks.

Given the chronic and progressive nature of ocular pemphigoid, relapse was not uncommon. Among those achieving complete remission, 28.6 % (4/14) experienced disease relapse. At time of publication, a significant portion of our patients (18, 56.0 %) remained in "partial response" category. In our cohort, patients who relapsed while on maximal doses JAKi were managed with the addition of an antimetabolite and/or low dose chronic prednisone, as clinically tolerated.

In conclusion, this case series contributes to the growing body of evidence supporting JAKi as a promising therapeutic option in the management of refractory ocular pemphigoid. Despite limitations, our findings suggest that JAKi therapy may achieve meaningful clinical improvement, decrease in patient treatment burden, and even remission in a cohort refractory to traditional therapies. Larger prospective studies are needed to further evaluate the long-term efficacy, safety, and optimal implementation of JAKi for ocular pemphigoid.

CRediT authorship contribution statement

Celine Nguyen: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Ebuka Eziama:** Writing – review & editing, Data curation. **Arturo R. Dominguez:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Jennifer H. Cao:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization.

IRB approval status

Reviewed and approved by the University of Texas Southwestern Medical Center IRB.

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Conflicts of interest

We have no relevant conflicts of interest to disclose.

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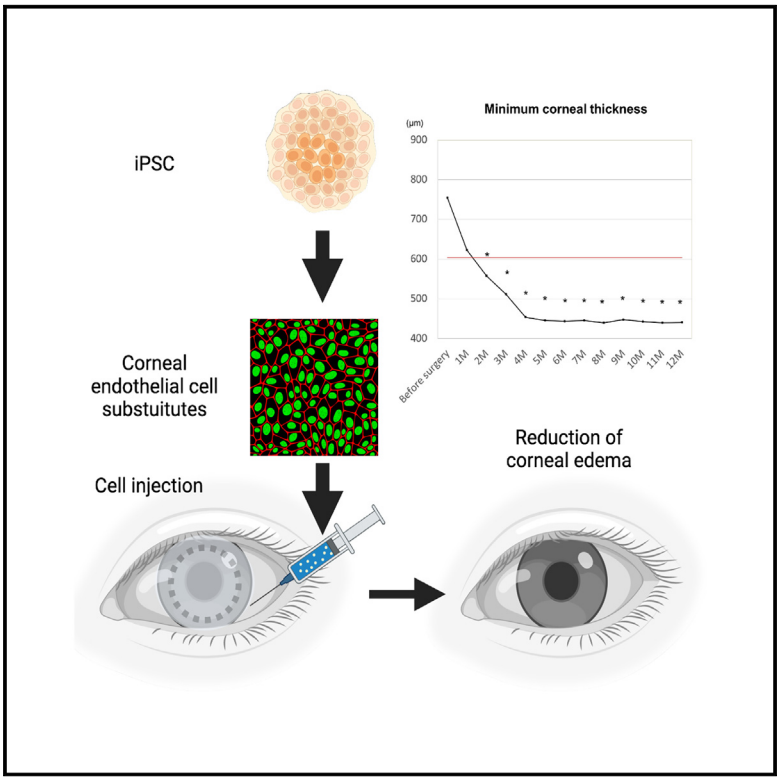
Report

A first-in-human clinical study of an allogenic iPSC-derived corneal endothelial cell substitute transplantation for bullous keratopathy

Graphical abstract



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In brief

Shimmura and colleagues demonstrate the feasibility of using iPSC-derived cells for treating bullous keratopathy. Although adverse reactions are not observed, *de novo* gene mutations can be detected despite extensive whole-genome sequencing of the master cell bank.

Highlights

- iPSC-derived corneal endothelial cell substitutes recover pathological corneal edema
- No adverse reactions are observed after 1 year follow-up
- Gene mutations may appear despite whole-genome sequencing of the master cell bank

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Report

A first-in-human clinical study of an allogenic iPSC-derived corneal endothelial cell substitute transplantation for bullous keratopathy

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SUMMARY

A first-in-human investigator-initiated clinical study of a corneal endothelial cell substitute (CLS001) derived from a clinical-grade induced pluripotent stem cell (iPSC) line shows improvement of visual acuity and corneal stromal edema, with no adverse events for up to 1 year after surgery for the treatment of bullous keratopathy. While preclinical tests, including multiple whole-genome analysis and tumorigenicity tests adhering to the Food and Drug Administration (FDA) draft guidelines, are negative, an additional whole-genome analysis conducted on transplanted CLS001 cells reveals a *de novo* in-frame deletion of exon22 in the *EP300* gene. No adverse events related to the mutation are observed. Our study demonstrates the feasibility of using iPSC-derived cells to replace donor transplant for bullous keratopathy, while shedding light on risk management of gene mutation in cell products. Further follow-up is required for long-term analysis of clinical safety and efficacy.

INTRODUCTION

The corneal endothelium is a single layer of cells located on the innermost surface of the cornea. It regulates the water content of the corneal stroma through its pumping and barrier functions and contributes to the maintenance of corneal transparency.[1] Corneal endothelial cell density (ECD) is approximately 2,000 cells/mm^2 in healthy individuals.[2] Human corneal endothelial cells lack the ability to regenerate *in vivo* after birth, and their number decreases as a result of aging, surgery, and genetic disease such as Fuchs' endothelial corneal dystrophy.[3–5] Corneal endothelial dysfunction due to progressive reduction of ECD causes bullous keratopathy, which is characterized by corneal edema and opacity. Bullous keratopathy has become the most common indication for corneal transplantation.[6,7] Although the number of patients waiting for corneal transplantation exceeds 12 million worldwide, the annual number of corneal transplants remains at approximately 180,000.[7] Therefore, a drastic solution to the chronic shortage of donors is needed.

Penetrating keratoplasty has a long history as a treatment for bullous keratopathy. However, there are risks of intraoperative and postoperative complications including immunological rejection, infection, and the development of glaucoma.[6] Over the past two decades, partial corneal transplantation techniques, such as corneal endothelial keratoplasty, have emerged. These procedures have helped to reduce the risk of intraoperative complications,[8] although bullous keratopathy may recur due to a gradual decrease in graft ECD.[9] In recent years, cultivated human corneal endothelial cell transplantation by anterior chamber cell injection has been reported as a novel treatment for bullous keratopathy.[10] The advantage of cell injection therapy is its reduced invasiveness compared to conventional keratoplasty. However, challenges remain in overcoming the donor shortage because cell sources rely on donor corneas and there are limitations on the number of cells that can be cultured from a single cornea.

In a previous study, we reported the development of corneal endothelial cell substitutes derived from human induced pluripotent stem cells (iPSCs) (CLS001). These cells were shown to have equivalent pump and barrier function to corneal endothelial cells *in vitro*.[11] In a preclinical study, the efficacy of cell injection therapy of CLS001 was demonstrated using a cynomolgus monkey (*Macaca fascicularis*) bullous keratopathy model.[12] In this clinical study, we assessed the safety and effectiveness of

transplanting CLS001 in a patient with recurrent bullous keratopathy. CLS001 was manufactured at the Cell Processing Center of Keio University Hospital (KHCPC), and quality control included detection of residual undifferentiated iPSCs, karyotyping, *in vitro* growth analysis, whole-genome sequence analysis, sterility testing, endotoxin testing, and mycoplasma and virus testing.

RESULTS

Patient characteristics

The original study plan was to enroll three patients between August 1, 2022, and March 30, 2023; however, the study ultimately enrolled only one patient for reasons described later. The patient was a 73-year-old male. His primary disease was keratoconus, for which his left eye received penetrating keratoplasty at another hospital 48 years ago. Following the surgery, the corneal endothelium of the graft gradually declined, leading to the diagnosis of bullous keratopathy. Consequently, the patient sought medical care at the Department of Ophthalmology at Keio University Hospital (herein referred to as "our hospital") eight years ago. Six years ago, he underwent regrafting of the left eye at our hospital followed by cataract surgery, and his postoperative clinical progress was favorable (best-corrected visual acuity [BCVA] 0.9 (20/22)).

Three years ago, he was diagnosed with mild endothelial rejection, ocular hypertension, and corneal infection, all of which improved with conservative treatment. However, he subsequently experienced a recurrence of bullous keratopathy, leading him to express interest in participating in this clinical study. Following the acquisition of informed consent, we conducted a preoperative eye examination on his left eye as part of visit 0, following the approved protocol. Briefly, in his left eye, BCVA was 0.02 (20/1,000), and intraocular pressure was 6 mmHg. Bullous keratopathy after regrafting was observed by slit-lamp examination, along with mild stromal opacification attributed to scarring following a previous corneal infection. His central corneal thickness was 863 μm, and minimum corneal thickness was 755 μm, which is thicker than the average central corneal thickness in normal Japanese of 531.7 μm.[13] Allogenic CLS001 was transplanted into the anterior chamber in the left eye of this patient.

Characteristics of the iPSC-derived corneal endothelial substitute cells

Prior to the clinical-grade production of CLS001, a separate batch of CLS001 was manufactured at KHCPC using the identical manufacturing process and was used for preclinical studies (Figure 1A). Two separate tumorigenicity tests were conducted, both of which did not show tumor formation. The first tumorigenicity study also serving as an overdose toxicity study done with 1×10^6 cells/eye, or 1,000 body weight equivalents per eye embedded in Matrigel, revealed sparse expression of the proliferating cell marker Ki67, and no tumorous growth was observed on histopathological examination after 52 weeks.

The second tumorigenicity study also served as a systemic distribution and toxicity test with 2.5×10^4 cells/eye, or 25 body weight equivalents per eye without Matrigel. Transplanted cells did not form tumors and eventually disappeared by slit-lamp microscopy at 16 weeks after transplantation. Cells did not attach to the cornea since the endothelial cells in animals were not scraped. After confirming that no cellular aggregates remained in the anterior chamber of rats by slit-lamp examination, 30 out of 36 rats were sacrificed for pathology. H&E staining, human cell marker Ku80 staining, and Ki67 staining did not reveal any residual transplanted cells in the pathology samples, and no other abnormal findings were observed (Figures 1B and 1C). The remaining 6 animals were sacrificed at 24 weeks after confirming that there was no repopulation of transplanted cells in the eye. Human Alu sequence PCR was performed on the transplanted eyes and major organs in these animals to confirm the absence of human DNA (Table S1). Hematology, blood biochemistry, and urinalysis performed during necropsy of animals also showed no abnormalities.

We used one lot of the CLS001 cells for *in vitro* quality tests. Immunostaining showed that CLS001 expressed Na, K-ATPase, tight junction formation by ZO-1 and adherence junction formation by N-cadherin, and the transcription factor PITX2 in the cell nucleus (Figure 1D). Confluent monolayer of CLS001 indicated their complete tight junction formation (Figure S1A). To assess cellular pump and intercellular junction formation, we conducted flow cytometry analysis to determine the percentages of Na, K-ATPase alpha-1 subunit-positive cells, mineralocorticoid receptor-positive cells, and N-cadherin-positive cells, which were found to be 97.8%, 99.4%, and 98.9%, respectively (Figure 1E). While specific markers for cornea endothelium do not exist, a combination of $CD166^{high}/CD105^{-}/CD44^{-}/CD26^{-}/CD24^{-}$ was previously reported.[14] CLS001 expression of these markers was $CD166^{high}/CD105^{-}/CD44^{low}/CD26^{-}/CD24^{high}$[14] (Figure S1B; Table S2).

We calculated the residual undifferentiated iPSC rate using quantitative PCR with the OCT4 relative expression rate as an indicator, and the findings indicated a rate of less than 0.01% (Figure S2). Additionally, we evaluated the presence of residual undifferentiated cells in CLS001 by an iPSC "back-culture test," where CLS001 cells were cultured for one week in 6-well plates with iPSCs medium (AK03N) and iMatrix-511 coating. Using this method, no iPSC colonies were detected in CLS001. (Figure S3). Karyotyping results were normal (Figure S4). Cell growth analysis demonstrated the absence of any proliferating cells (Figure S5). All sterility tests, endotoxin tests, and mycoplasma and virus negativity tests were normal. For shipment, the number of viable cells was adjusted to 10×10^5 cells/200 μL, and 8×10^5 cells/160 μL was administered to the subject. Cell viability rate was 91.2%. Insoluble foreign matter test, sterility test, and endotoxin test were all normal. The results and standard values for each test are summarized in Table S3. The preservation period of frozen CLS001 was set at 2 months based on these preclinical data.

Primary outcomes

Cell injection was successfully performed without major complications (Figures 2A and 2B). We did not observe any adverse events including unintended cell proliferation or cell mass in the vitreous cavity, intraocular pressure increase, and rejection during the entire follow-up time course (Figure 3). We also

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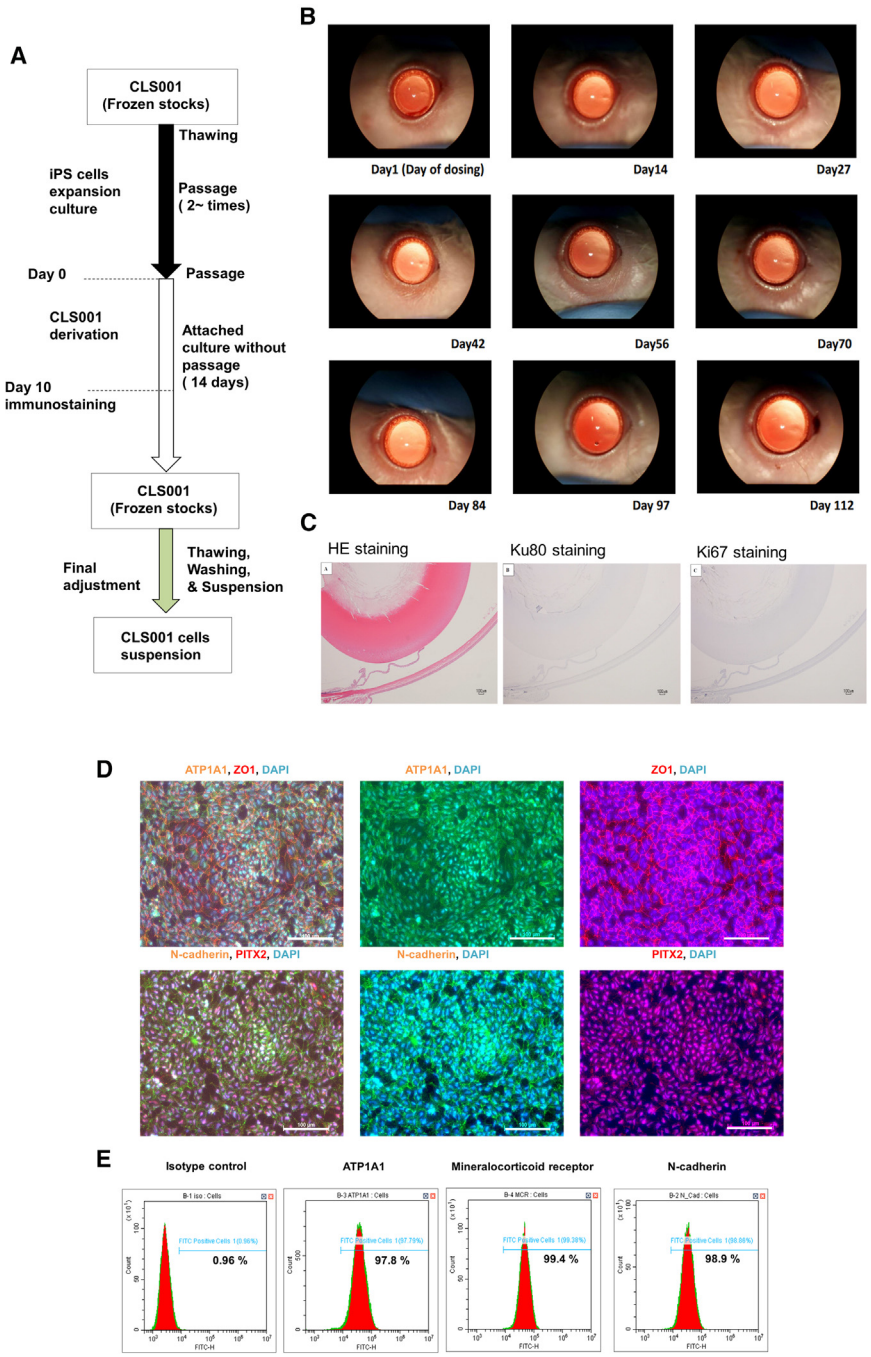


Figure 1. CLS001 derivation from iPSCs

(A) Process flow of CLS001 derivation from iPS cells, cryopreservation, and final adjustment of CLS001 cells suspension. After initial expansion culture of iPS cells, CLS001 cells were differentiated from iPS cells.

(B) Representative time course images of anterior chamber after cell injection.

(C) Pathological examination of H&E staining, human cell marker Ku80 staining, and proliferating cell marker Ki67 staining to reveal no residual transplanted cells after cell injection.

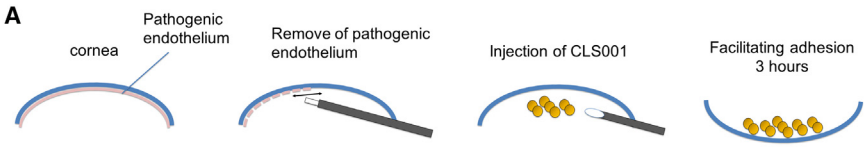
(D) Immunostaining of CLS001. CLS001 expressed Na, K-ATPase alpha-1 subunit (ATP1A1), linear ZO1 and N-cadherin, and nuclear PITX2. Scale bars, 100 μm.

(E) Flow cytometry analysis of CLS001 cells after thawing is shown. Expression rate of ATP1A1, mineralocorticoid receptor, and N-cadherin of CLS001 was above 90%.

confirmed that there were no adverse events caused by surgery and procedures such as infection and expulsive hemorrhage during/after surgery. In the final medical examination of the 1-year course, a urine test revealed asymptomatic occult blood in the urine. The patient was then referred to the urology department, where no specific abnormalities were found. Residual host Descemet's membrane was observed post-operatively on anterior segment optical coherence tomography (AS-OCT) examination, but it was located in the periphery and had no effect on visual function. Brown pigmentation, which was attributed to the adhered injected cells, was observed on the corneal endothelial surface. However, this observation did not lead to any specific adverse events or complications including the ocular fundus (Figures 3A and 3B). Corneal ECD was unmeasurable by non-contact specular microscopic examination throughout the study (Figure 3B).

Secondary outcomes

A gradual improvement in BCVA was observed at every visit after 3 months, including the final evaluation conducted 1 year after cell injection. (Figure 3). The subject's preoperative corrected visual acuity was 0.02 (20/1,000) (uncorrectable). Corrected visual acuity with hard contact lens (HCL) wear was not measured preoperatively. After surgery, the BCVA improved to 0.07 (20/286) with spectacles and 0.5 (20/100) with HCL (Figures 3A and 3C). In the AS-OCT, the color-coded map of corneal pachymetry showed noticeable improvement in central and thinnest corneal thickness during follow-up. The pathologic corneal thickness (purple or blue) improved after cell injection. The scarred area in the lower central region of the cornea due to infectious keratitis was thinner than the surrounding area before surgery, and a similar trend was observed as edema improved following cell injection (orange) (Figure 3A). In the cross-sectional images (inferior part of cornea shown on the left, superior part on the right) also shows decrease in the thickness of the corneal stroma. No mass formation due to cell proliferation



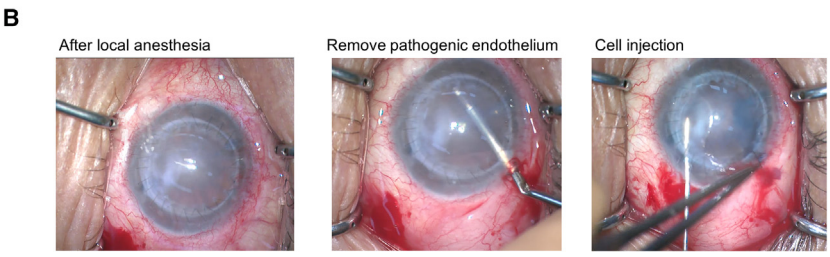


Figure 2. Surgical procedures of cell injection of CLS001

(A) Schematic presentation of surgical procedures of CLS001 cell injection therapy.

(B) Representative images of performed surgery.

was observed on the endothelial surface, angle, or other areas of the cornea. The area of thinning observed in the lower (left) region compared to the surrounding area in the cross-sectional image indicates scar formation due to preoperative infectious keratitis, which is consistent with the pachymetry results (Figure 3A). The endothelial surface where cells are presumed to have adhered was relatively brighter than the surrounding area on AS-OCT. This, together with the pigmentation on the endothelial layer on slit-lamp examination, and the difficulty in observing the cell structure in specular microscopy (Figure 3B), suggests that the morphology of cells may be different from the normal structure of the corneal endothelium.

De novo EP300 gene mutation in cells

In a preclinical study using the same passage number cells as in this clinical study, whole-genome analysis showed no abnormalities in both the QHJI01s04 iPS cells or the differentiated CLS001 cells. Furthermore, we confirmed that there were no abnormalities in the whole-genome analysis of the QHJI01s04 master cell bank (MCB) lot to be used for this clinical study as well. The timing of whole-genome analysis adhered to the notification set by the Japanese Ministry of Health, Labor and Welfare and complied with the Food and Drug Administration (FDA) draft guideline (https://www.fda.gov/media/178113/download) posted after this study. Under the guidance of the Keio Specific Certified Regenerative Medicine Committee, whole-genome analysis was performed once again on the CLS001 cells transplanted to the patient to ensure maximum safety, although the results would not be known until after administration due to the limited cryopreservation period of the cells. Furthermore, measures to be taken if the results of whole-genome analysis revealed abnormalities in the administered cells were also clearly stated in advance in the clinical research protocol and informed consents (IC) to subjects.

The final whole-genome analysis revealed a deletion on the *EP300* gene, which is listed in the Cancer Gene Census.[15] Specifically, nucleotides on chr22:41,559,981–41,560,191 (hg19), where exon 22 of *EP300* gene is located, were deleted (Figure 4). The deletion was validated by droplet digital PCR (ddPCR), and its variant allele frequency (VAF) was 36.1%. Even though exon 22 is skipped in spliced mRNA, exon 21 connects to exon 23 in-frame, which was confirmed by RNA sequencing (RNA-seq) (Figure S6A). Zygosity of the deletion was confirmed through single-cell cloning. Reverse-transcription PCR (RT-PCR) of the *EP300* deletion site revealed two expression patterns: colonies with both mutant and WT bands (total 16 colonies) and colonies with no mutant bands (total 4 colonies) were isolated, but there were no colonies that showed only the mutant *EP300* band expression (Figure S7). Thus, we concluded that this *EP300* mutation was heterozygous.

The quantitative PCR analysis developed for this study showed that the contamination rate of cells with *EP300* mutation was 6.3% at the MCB stage, and the contamination rate increased with each expansion culture, resulting in a contamination rate of 79.2% in the final product. When CLS001 was re-manufactured from the same MCB lot using the same manufacturing method, mutant cell contamination rate increased with each passage (Table S4). This suggests that the mutation did not emerge *de novo* during the CLS001 differentiation induction process but rather resulted from the expansion culture of the MCB.

After reviewing the initial findings on the causes and countermeasures for the mutation, the Specific Certified Regenerative Medicine Committee agreed to increase the frequency of follow-up observations for 1 year post-surgery to enhance patient safety, adhering to the guidelines of the approved research protocol. Additionally, concerning the enrollment of future patients, the committee emphasized the importance of verifying whole-genome analysis results prior to surgery to ensure safety. However, to address the cause of mutation, quality test using a different MCB lot was required, a process not feasible within the original patient enrollment time frame. Consequently, the decision was made to conclude the study with only one participant. A final report was submitted to the Ministry of Health, Labor and Welfare to conclude the study.

This individual was monitored monthly, and the 1-year follow-up was successfully completed as originally planned. No adverse events were observed in the subject. After detecting the *EP300* gene mutation, we conducted follow-up experiments to evaluate the differentiation ability, cell growth, and RNA expression of MCB iPSCs. We used 3 lots of iPSCs (15M48, 15M49, and 15M50), whose VAFs were approximately 0%, 50%, and 10% (n = 3 each) at passage 19, respectively, as determined by the results of ddPCR analysis (Table S5). No irregularities in iPSC morphology were detected in any of the

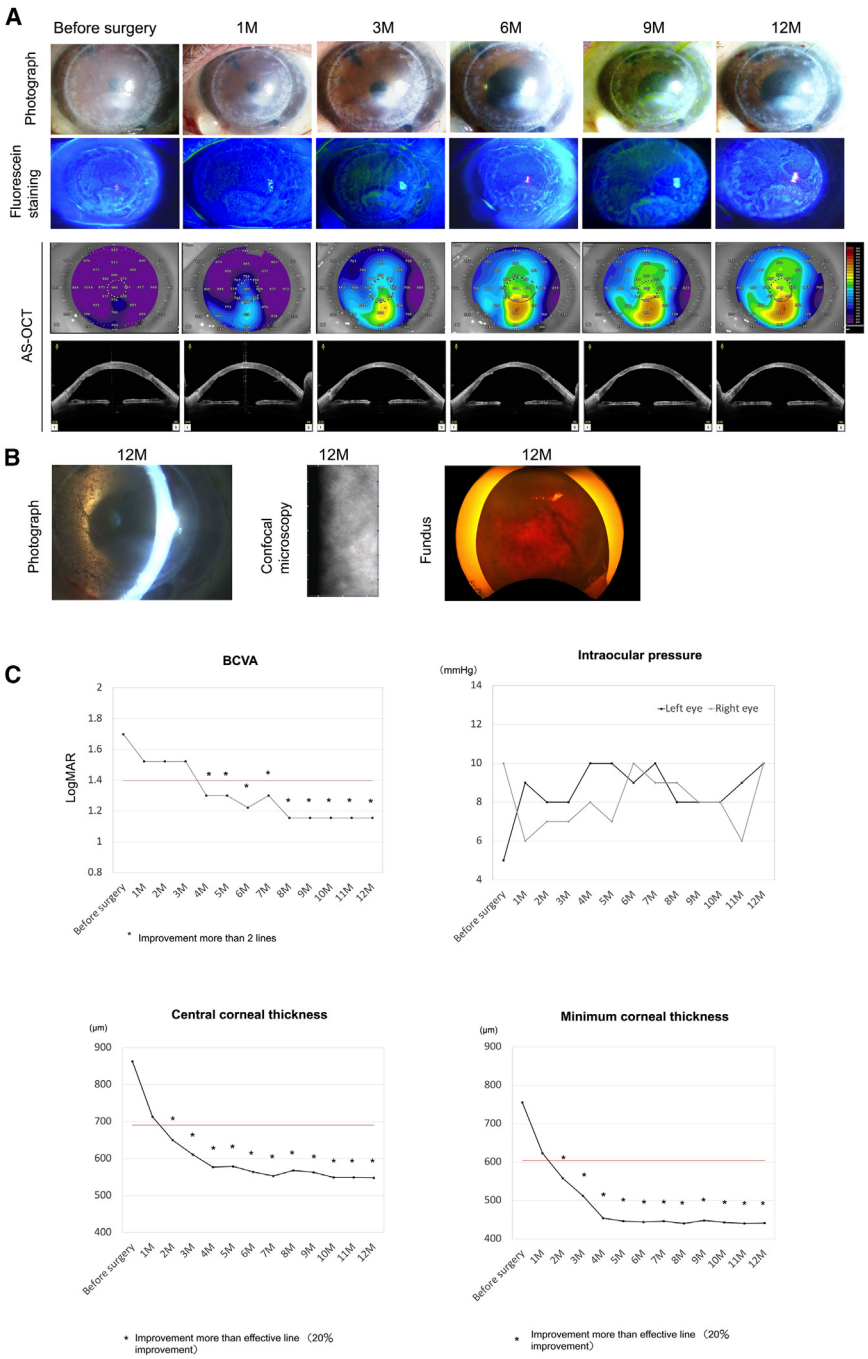


Figure 3. Clinical time course follow-up of eye examinations after cell injection therapy

(A) Representative images of 1-year clinical time courses of slit-lamp examination, fluorescein staining, and anterior segmental OCT after surgery.

(B) Representative images of slit-lamp examination, confocal microscopy, and fundus photograph at last visit examination.

(C) Best corrected visual acuity, intraocular pressure, and corneal thickness. Measures of best-corrected visual acuity (BCVA), intraocular pressure, and corneal thickness are provided. The results with respect to the secondary outcomes of a corneal thickness of less than 20% improvement and an improvement in BCVA of two lines or more on a Landolt C eye chart (a measure of decimal visual acuity) as compared with baseline were marked as asterisk (*). BCVA is expressed as logMAR visual acuity. Intraocular pressure was within normal range during each evaluation period.

samples at passage 19 (Figure S8). No deficiencies of differentiation ability to endoderm, mesoderm, and ectoderm were observed (Figure S9). While the doubling times were within standards, one sample with the EP300 mutation seemed to be slightly shorter (Table S6). The enrichment analysis including gene ontology (GO) and pathway analysis of mutated cells showed enhanced expression of genes related to cell cycle and gene expression, which is consistent with the slightly enhanced doubling time and *EP300* gene function (Figures S6B–S6E). In order to evaluate whether the mutation has any effect on the barrier and pump function of CLS001, we obtained a different MCB of the same QHJI01s04 iPS cell line used in the clinical study, with the same passage number, but with a lower contamination of EP300 mutant cells. Using both the original MCB used in the clinical study and the newly acquired MCB, we induced CLS001 into three different lots. We evaluated pump function and barrier function using ZO-1 immunostaining (Figure S10) as surrogate markers and by flow cytometry for ATP1A1 and N-cadherin (Table S7). In both immunostaining images, linear ZO-1 expression was observed along the cell boundaries, and there was no difference in the expression rates of ATP1A1 and N-cadherin.

We are conducting a follow-up clinical research study approved by the institutional review board to observe the patient for up to the fifth year after cell transplantation. Clinical data include visual acuity, intraocular pressure, corneal topography analysis, and corneal and conjunctival findings, including corneal ECD. Additional examinations can be performed at the patient's request. There are no adverse effects observed up to the time of submission.

DISCUSSION

We report a first-in-human clinical study of bullous keratopathy treated with corneal endothelial substitute cells (CLS001)

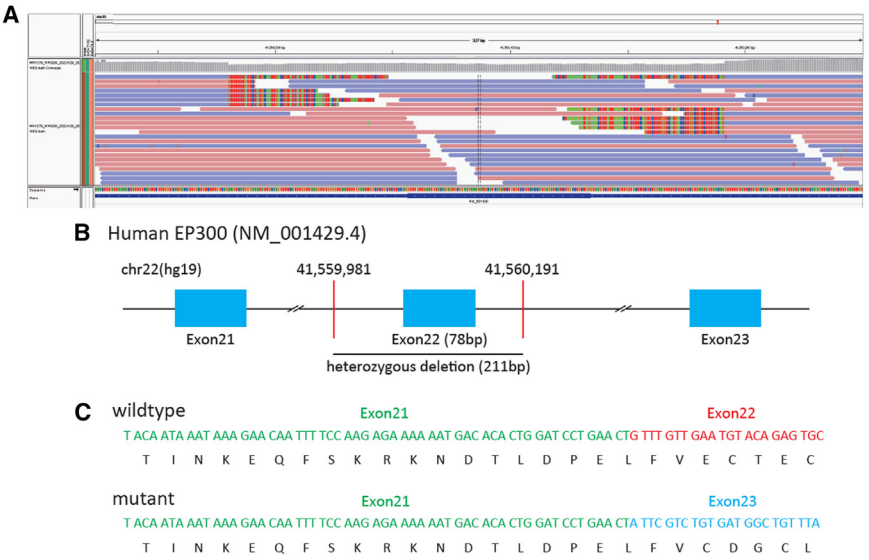


Figure 4. The whole-genome analysis with cells of the same lot revealed a deletion on the gene *EP300* listed in the Cancer Gene Census

(A) Sequenced reads obtained by whole-genome sequencing and mapped to loci around exon 22 of *EP300* gene were visualized using the Integrative Genome Viewer (IGV).[16] Soft-clipped sequences were colored in accordance with their nucleotides.

(B) The deletion detected by whole-genome sequencing analysis was illustrated.

(C) The nucleotide sequences and amino acid sequences in wild-type and estimated ones in mutant were shown.

derived from iPSCs. There were no adverse effects observed following transplantation, neither at the time of surgery nor during the subsequent clinical follow-up. Pigmentation of the cells was observed by slit lamp starting at 3 months following surgery, and observation of the corneal endothelium surface by AS-OCT showed higher reflectivity compared to normal corneal endothelium.

Evaluating cell morphology and density by specular microscope was not possible due to the preoperative scar, as well as subtle differences in the endothelial surface, which may have played a role. While functional recovery of corneal thickness was achieved, further observation will be required to monitor the impact of the difference in clinical phenotype observed by slit-lamp examination and AS-OCT. We have set up an observational clinical study after this study to carefully follow up the clinical progress. The study protocol also allows for the patient to undergo a standard corneal transplantation upon request, at which time a more detailed pathological analysis would be possible.

Despite improvement in corneal edema and increased transparency, improvement in corrected visual acuity was modest. Visual acuity improved by applying an HCL indicating that irregular astigmatism due to scarring was partially responsible. Further studies in milder cases of bullous keratopathy will be required for evaluating the efficacy of CLS001.

There were no signs of tumor formation, a concern specific to the use of iPSCs. In compliance to current recommendations set by the FDA and the Japanese Ministry of Health, Labor and Welfare, whole-genome analysis of MCB and CLS001 was repeated three times prior to clinical use, which did not reveal any abnormalities. Under the guidance of the Keio Specific Certified Regenerative Medicine Committee, a fourth whole-genome analysis was performed on CLS001 cells, although the results of the last test would not be known until after administration due to the limited cryopreservation period of the cells. In order to prioritize safety of the current study, we limited the number of cases to only one patient. We also established a systematic method to quantify mutations in cell stocks, which can be applied to quality control of iPSCs for future applications. During the follow-up period, no adverse events attributable to the *EP300* gene mutation were observed. This series of events was immediately reported to authorities, including the Keio University Specific Certified Regenerative Medicine Committee, and countermeasures were set under their guidance.

Upon further investigation of CLS001, an in-frame deletion of exon22 in *EP300* gene was identified,[17] which would cause heterozygous loss of the Plant Homeodomain (PHD) finger in p300 protein. This specific mutation was not listed in the Catalogue of Somatic Mutations in Cancer. While the mutation was not detected during whole-genome analysis of QHJI01s04 MCB used in this clinical study, quantitative PCR revealed that these mutations did not originate during the differentiation process of CLS001 but had increased during expansion culture of the iPSCs. Doubling times of samples with the mutation seemed to be slightly shorter, which was consistent with the finding of the enrichment analyses (Figure S6E).

In addition to repeated whole genomic analysis in the preclinical studies, we conducted *in vivo* tumorigenicity test in both CLS001 cells for preclinical studies and clinical-grade CLS001 cells manufactured in KHCPC, both of which did not show tumorigenicity. We used the anterior chamber of the eye for the tumorigenicity test, which is a sensitive tumorigenicity test since direct observation is possible through the clear cornea.[18] Given the absence of abnormalities observed in the *in vivo* tumorigenicity test that included cells with *EP300* mutations, it appears that this specific mutation has no substantial impact on the tumorigenicity in differentiated cells, especially if they do not proliferate as in the case with CLS001 cells. Avoiding all mutations may prove to be difficult due to sensitivity of whole-genome testing. Since we still do not fully understand the implications of such random mutations, nor the risks involved, further discussion is required on how to handle such event in developing cell products from pluripotent stem cells.

In conclusion, CLS001 was transplanted into a patient with severe bullous keratopathy, and partial improvement of corneal edema, transparency, and visual acuity were observed. Since removal of diseased corneal endothelium alone will not improve corneal edema and transparency, the reduction in corneal

thickness can be attributed to the injected cells. No clinical adverse events were observed, although pigmentation of transplanted cells and high reflectivity on AS-OCT images suggested a slightly different phenotype from normal corneal endothelium. Furthermore, the detection of a *de novo* gene mutation indicates that such mutations can occur despite repeated preclinical testing. Current guidelines set by the FDA may not be sufficient for first-in-human studies, suggesting the need for a more comprehensive strategy in iPSC-based cell therapy. Furthermore, the implications of such potentially nonfunctional mutations in tumor-related genes require further discussion by specialists in the field.

Limitations of the study

There are several limitations to this study. First, despite adhering to the latest safety guidelines on cell therapy, a *de novo* mutation was detected in a redundant whole-genome test. While there were no adverse effects observed in the patient, the study did not recruit the two additional patients intended in the original study design. Second, ZO-1 immunostaining was used a surrogate marker for tight junction function since transepithelial electrical resistance measurement was not logistically possible on cells to be used in patients. Third, analysis of the surface markers of CLS001 revealed CD44-positive cells in approximately 20% of the cells. CD44 is reported to be a negative marker for mature corneal endothelial cells,[14] suggesting that there may be precursor cells remaining, but the impact of this is unknown. Since the cell growth assay showed that the cells were not proliferating, we do not consider this to be a safety issue.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Shigeto Shimmura (shigeto.shimmura@fujita-hu.ac.jp).

Materials availability

This study did not generate new unique reagents.

There are restrictions to the availability of QHJI01s04 iPS cells MCB. The QHJI01s04 iPS cells used in this study will be made available from CiRA Foundation on request under a collaborative research agreement.

Data and code availability

- The datasets used in this study include whole-genome sequencing (WGS) and RNA-seq data. The raw sequencing data from this study are not publicly available due to patient and/or donor privacy regulations. To request access to the raw data, please contact the lead contact at shigeto.shimmura@fujita-hu.ac.jp. The data are available through the European Genome-Phenome Archive (EGA) under accession number EGAS50000000672 (WGS) and EGAS50000000673 (RNA-seq). The request will be reviewed by the Data Access Committee of CiRA Foundation (EGAC50000000128). A request for the raw data must include the objectives of the research project for which the data will be used. Data access will be permitted for the purpose of research developing regenerative medicine or regenerative cell therapy. The person, who would obtain permit, acknowledges that they will have access to personally identifiable information or sensitive clinical information and such requests for the raw data access must rigorously adhere to the consent agreements established with donors of the iPSCs. Such requests must be accompanied by institutional approvals.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.S. and S.H. Methodology: S.S., S.H., M.H., and T.H. Investigation: S.S., S.H., M.H., R.H., O.I.H., H.D., K.A., M.N., N.T., T.H., T.S., and E.I. Funding acquisition: S.S. and S.H. Project administration: S.S., S.H., H.M., and K.N. Supervision: S.S., T.H., H.O., and K.N. Writing – original draft: M.H., S.H., H.D., and M.N. Writing – review and editing: S.S., S.H., N.T., T.H., and H.O.

DECLARATION OF INTERESTS

S.S. and S.H. have a patent on CLS001 differentiation protocol.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Polyclonal Anti-PITX2 - N-terminal region	AVIVA SYSTEMS BIOLOGY	Cat#ARP32431_P050; RRID:AB_2047532
Mouse monoclonal anti human n-cadherin	Invitrogen	Cat#33-3900; RRID:AB_2313779
Rabbit Polyclonal anti-human ZO1	Invitrogen	Cat#40-2200; RRID:AB_2533456
Mouse monoclonal anti human Na,K-ATPase alpha 1 subunit	NOVUS	Cat# NB300-146; RRID:AB_2060981
Mouse monoclonal Anti Na+/K + -ATPase α1 (C464.6), Alexa Fluor 488	SANTA CRUZ	Cat#SC-21712-AF488; RRID:AB_626713
Mouse monoclonal anti-MCR (H10E4C9F), Alexa Fluor 488	SANTA CRUZ	Cat#SC-53000- AF488; RRID:AB_784896
Mouse monoclonal anti-human CD325 (N-Cadherin) antibody clone:MC-813-70, Alexa Fluor 488	Biolegend	Cat#350810; RRID:AB_11219390
Ku80 (C48E7) Rabbit mAb	Cell Signaling Technology Inc.,	Cat#2180; RRID:AB_2218736
Ki67 anibody [SP6]	GeneTex Inc.,	Cat#GTX16667; RRID:AB_422351
Sox17 Antibody, anti-human, PE, REAfinity	Milteny Biotec	Cat#130-111-032; RRID:AB_2653493
FoxA2 Antibody, anti-human, APC, REAfinity	Milteny Biotec	Cat#130-123-850; RRID:AB_2819525
Anti-Human CD56(NCAM) Antibody, APC	STEMCELL Technologies	Cat#60021AZ; RRID:AB_2938621
Brachyury (D2Z3J) Rabbit mAb,Alexa Fluor® 488 Conjugate	CST	Cat#94663S; RRID:AB_2799983
PE anti-Nestin Antibody	BioLegend	Cat#656806; RRID:AB_2566382
PAX-6 Antibody, anti-human, APC, REAfinity	Milteny Biotec	Cat#130-123-267; RRID:AB_2819462
Chemicals, peptides, and recombinant proteins		
Stemfit AK03N	AJINOMOTO HEALTHY SUPPLY CO., INC.	N/A
iMatrix-511MG	MATRIXOME	Cat#892005
iMatrix-511	MATRIXOME	Cat#892012
Y-27632	FUJIFILM Wako Pure Chemical Corporation	Cat#039-24591
Corneal Endothelial Differentiation Medium	Hatou et al.[13]	N/A
DMEM/F-12	Thermo Fisher Scientific Inc.	Cat#11330-032
ITS (Insulin, transferrin, selenium) - Animal Free	InVitria	Cat#777ITS091
Somazon 10mg for Injection	Orphan Pacific	JANcode #4987858100075
HYDROCORTONE® Injection	Nichi-Iko Pharmaceutical	JANcode #4987376231114
LIF	FUJIFILM Wako Pure Chemical Corporation	Cat#125-06661
IL-11	PeproTech	Cat#AF-200-11 (10μg)
IL-6	Miltenyi Biotec	Cat#170-076-161
TNF alpha	Miltenyi Biotec	Cat#170-076-178
Vision Blue	D.O.R.C. International	Cat#46136-54673
BSS	Alcon	YJcode #1319731Q2045

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
0.1% betamethasone	Rohto Nitten	YJcode #1315706Q2129
1.5% levofloxacin	Rohto Nitten	YJcode #1319742Q2183
Critical commercial assays		
RNeasy Mini Kit	QIAGEN	Cat#74104
THUNDERBIRD SYBR qPCR Mix	TOYOBO	Cat#QPS-201
ReverTra Ace	TOYOBO	Cat#TRT-101
KAPA Hyper Prep Kit	Kapa Biosystems	Cat#KK8505
Infinium OmniExpress-24 v1.3 Kit	Illumina	20024631
2×ddPCR supermix for probes (no dUTP)	Bio-Rad	Cat#186-3023
HindIII	NEB	Cat#R0104S
STEMdiff Trilineage Differentiation Kit	STEMCELL TECHNOLOGIES	Cat#05230
TruSeq Stranded Total RNA Library Prep Gold	Illumina	Cat#20020598
Deposited data		
WGS and RNA-seq	This manuscript	EGA accession number: EGAS50000000672 (WGS), EGAS50000000673 (RNA-seq)
Experimental models: Cell lines		
Human: iPS cell line QHJI01s04	CiRA Foundation	N/A
Human: HCEC-B4G12	Leibniz Institute DSMZ	ACC 647
Experimental models: Organisms/strains		
Nude rats: F344/NJcl-rnu/rnu	CLEA Japan, Inc.	N/A
Oligonucleotides		
EP300 mutation RT-PCR primer	Table S8A	N/A
EP300 mutation qRT-PCR primer	Table S8B	N/A
EP300 endogenous control qRT-PCR primer	Table S8B	N/A
EP300 ddPCR primer	Table S8C	N/A
EP300 wild-type ddPCR probe	Table S8D	N/A
EP300 mutation ddPCR probe	Table S8D	N/A
POU5F1 (*OCT4*) qPCR primer	Table S8B	N/A
Software and algorithms		
Step One software, version 2.3	Applied Biosystems	https://stepone-software.software.informer.com/2.3/
bcl2fastq v2.20.0.422	Illumina	https://support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html
fastp v0.20.1	Chen et al.[19]	https://github.com/OpenGene/fastp
GenomeStudio 2.0.4	Illumina	https://support.illumina.com/array/array_software/genomestudio/downloads.html
QuantaSoft 1.7.4	Bio-Rad	bio-rad.com
ENCODE long-rna-seq-pipeline 2.3.4	ENCODE project	https://www.encodeproject.org/pipelines/ENCPL002LPE
R v4.2.3	R Core Team	https://www.R-project.org
DESeq2 v1.38.3	Love et al.[20]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Enrichr	Xie et al.[21–23]	https://maayanlab.cloud/Enrichr/

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Integrative Genome Viewer 2.16.1	Robinson et al.[16]	https://igv.org/doc/desktop/#DownloadPage/
Other		
OCT (CASIA2)	Tomey Corporation	https://www.tomey.de/products/casia2
specular microscopy	Tomey Corporation	EM-3000
iCare TOMOMETER	M.E. Technica	iCare IC200
NovaSeq6000	Illumina	Cat#20012850
QX200 Droplet Digital PCR System	Bio-Rad	Cat#1864001J2

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participant

A 73 years-old Asian male with a history of previous keratoplasty was recruited for the study. This study was conducted in accordance with the Act on Ensuring the Safety of Regenerative Medicine, etc., a law enacted in Japan. Under this system, research plans are reviewed by the Ministry of Health, Labor and Welfare's Health Sciences Council (Regenerative Medicine Evaluation Subcommittee) based on extensive preclinical works with iPS-derived corneal endothelial substitute cells. The study plan and protocol were approved by the Specific Certified Regenerative Medicine Committee at Keio University Hospital. The progress of the research was continuously monitored by both of Ministries and Agency and Committee, and any amendments to the research plan required approval. This research plan was also reviewed by AMED (Japan Agency for Medical Research and Development), which is under the jurisdiction of the Cabinet Office along with the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Ministry of Economy, Trade and Industry. We planned a nonrandomized, single-group study involving a small number of participants (maximum 3 cases). The protocol and other relevant study documentation of this clinical study were reviewed for compliance with the guidelines on clinical research with human stem cells in Japan, and were approved by the Institutional Review Board and Specific Certified Committee for Regenerative Medicine at Keio University School of Medicine, and by the Specific Committee of the Japanese Ministry of Health, Labor, and Welfare. The authors vouch for the accuracy and completeness of the data and analyses and the reporting of adverse events and for the fidelity of the study to the protocol.

Animal studies

Four-week-old male and female nude rats (F344/NJcl-rnu/rnu) were used for pre-clinical studies. Mice were maintained under a controlled environment with a 12-h light/dark cycle, a temperature range of 20.7°C–23.2°C, and relative humidity between 37 and 68%. Mice were fed a standard laboratory diet (CRF-1, Oriental Yeast Co, Tokyo, Japan) and had *ad libitum* access to water. Animals were housed at 2 to 3 animals/cage. In accordance with the guidelines outlined by the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research, all animals involved in the preclinical study were treated with the utmost care and ethical consideration. This study was approved by the Animal Research Committee of the School of Medicine of Keio University (Approval Number:15084) and also by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (Approval No. IACUC656-008).

METHOD DETAILS

Cell production

Corneal endothelial cell substitute from iPS cells (CLS001) was manufactured at KHCPC according to a previously described method.[12] Master cell bank (MCB) of clinical grade iPS cell line QHJI01s04 was purchased from Center for iPS Cell Research and Application Foundation (Kyoto, Japan) and subjected to genome analysis and sterility tests. This MCB is manufactured in compliance with good manufacturing practice (GMP) and tested according to ICH Q5 guidelines.[24] Briefly, a vial of MCB was thawed, disseminated on laminin-511-e8-fragment (iMatrix-511MG, Matrixome, Osaka, Japan) coated culture plates, and expanded with the Stemfit AK03N (Ajinomoto Healthy Supply, Tokyo, Japan) culture medium for three weeks. Subsequently, CLS001 was differentiated from expanded iPS cells on iMatrix-511MG coated culture dishes with Corneal Endothelial Differentiation Medium.[12] After 2 weeks of differentiation culture, CLS001 cells were collected from culture dishes and cryopreserved until the day of surgery. The cells were stored for a period of 2 months, and their stability during this time frame was previously verified through a quality test as outlined below, a result consistent with our earlier findings in the preclinical study. On the day of surgery, cells were thawed, washed by centrifugation, mixed with Rho-associated protein kinase inhibitor (ROCKi, Y27632, FUJIFILM Wako chemicals, Osaka, Japan) at a final concentration of 100 μM, adjusted to 5×10^7 cells/mL in KHCPC, and refrigerated until immediately before administration (Figure 1A).

Quality tests

Quality tests of frozen stocks of CLS001 cells (working cell bank, WCB) included flow cytometry analysis of corneal endothelial cell markers related pump function (Na, K-ATPase alpha-1 subunit, mineralocorticoid receptor) and intercellular junction (N-cadherin), detection of residual undifferentiated iPS cells, karyotyping, cell growth analysis, whole genome sequence analysis, sterility testing, endotoxin testing, mycoplasma and virus negativity testing. The methods and standard values for each test are summarized in Supplementary appendix and Table S3. Of these procedures, both cell growth analysis and whole genome sequence analysis required over two months for testing, and the outcomes were only revealed after the cells had been administered to the subjects.

Prior to shipping the cells for surgery, the cells were subjected to viability, sterility and endotoxin tests, as well as examining the vial for the presence of insoluble foreign matter. Of these, sterility tests were conducted twice; once on the cryopreserved WCB and the other on the culture supernatant of CLS001 on the day of surgery. The results of the WCB were known prior to administration to the subject and were negative. For the sample submitted on the day of surgery, the sterility test results were known after administration, and the results were also negative.

Preclinical safety test

We performed preclinical tests to evaluate tumorigenicity, systemic distribution, and systemic toxicity of CLS001 administered in the anterior chamber, the site of cell administration in clinical study.[18] We used 4-week-old nude rats (F344/NJcl-rnu/rnu) weighing approximately 54 g for this study. The endpoint of the anterior chamber tumorigenicity model was when the tumors macroscopically occupied the entire volume of the anterior chamber accompanied by notable protrusion of the eye compared to the fellow eye.[18]

The first tumorigenicity study was conducted by administering CLS001 into eyes of nude rats ($n = 32$) at a dose of 1×10^6 cells/eye, or 1,000 body weight equivalents per eye, and followed for up to 52 weeks or until the rats died of natural causes. Prior to the start of the clinical study, a second tumorigenicity study, also serving as a systemic distribution and systemic toxicity tests was conducted. In this case, the cells were administered into eyes of nude rats ($n = 36$) at a dose of 2.5×10^4 cells/eye, or 25 body weight equivalents per eye. Animals were followed for a maximum of 24 weeks, or until the cells disappeared from the anterior chamber. If cells were no longer discernible in the anterior chamber at a given time based on anterior segment photographs, observation was continued for an additional 4 weeks before judging that the transplanted cells have disappeared. Necropsy was conducted following the completion of the follow-up period.

Human Alu sequence PCR was performed on the transplanted eyes and major organs* in 6 animals to confirm the absence of human DNA. (*The following organs were examined: blood, lung (right anterior lobe), esophagus, stomach, colon, pancreas, liver (lateral left lobe), heart, kidney, bladder, testis, prostate, ovary, uterus, cerebrum, cerebellum, medulla oblongata, spinal cord (lumbar region), bone and marrow (sternum, femur), submandibular lymph nodes, spleen.) Hematology, blood biochemistry, and urinalysis were performed at necropsy on all animals.

Patient eligibility

Eligible patients fulfilled all of the following criteria

1. Have a diagnosis of bullous keratopathy following corneal transplantation, including a history of at least one penetrating keratoplasty.
2. Be aged 45 to 85 years at the time of providing informed consent, regardless of gender.
3. Exhibit a central corneal endothelial cell density of less than 500 cells/mm^2 or no detectable cells as determined by specular microscopy or confocal microscopy.
4. Be capable of providing voluntary consent to participate in the study.

Patients meeting any of the following conditions were excluded. However, conditions 1 to 4, 6, and 8 specifically pertain to the eye intended for transplantation.

1. Patients with keratoconjunctival disease of unknown etiology.
2. Patients with a central corneal thickness of 1,200 μm or greater, or evidence of vascular invasion into the corneal stroma.
3. Patients with either (a) active corneal infection or (b) systemic infection (bacterial, fungal, HBV, HCV, etc. virus-positive, etc.).
4. Patients with intraocular pressure of 30 mmHg or higher (Patients whose intraocular pressure is controlled to less than 21 mmHg with antiglaucoma medications were not excluded).
5. Patients with poorly controlled diabetes (HbA1C 8.0% or higher).
6. Patients with neovascularization in the angle or post-treatment neovascular glaucoma.
7. Use of specific medications during the perioperative and postoperative observation period, including anesthetics (xylocaine injection), antibiotics (1.5% levofloxacin hydrate), steroids (0.1% betamethasone sodium phosphate, 0.1% fluorometholone), and patients with a history of hypersensitivity to glaucoma drugs (prostaglandin preparations, beta blockers, etc.).
8. Patients planning to undergo intraocular surgery during the clinical study period.
9. Patients with a history of cancer.
10. Patients with severe liver impairment (AST >100 IU/L or ALT >100 IU/L).
11. Patients with severe renal impairment (serum creatinine level 1.5 mg/d or higher) requiring dialysis.

12. Patients with systolic blood pressure of 180 mmHg or higher or diastolic blood pressure of 110 mmHg or higher even with antihypertensive drugs.
13. Pregnant women, women who may become pregnant, or those planning to become pregnant during the clinical study period, or men wishing to impregnate their partner.
14. Patients unable to tolerate eye surgery under local anesthesia (e.g., severe claustrophobia).
15. Patients who have participated in other clinical trials or clinical research within 1 month before providing informed consent.
16. Patients deemed unsuitable for this clinical study due to complications or other factors.

Surgical procedure and postoperative follow-up

On the day of surgery, cryopreserved CLS001 WCB were thawed in KHCPC, adjusted to a cell density of 8 × 10^5cells/160 μL for administration, and refrigerated until shipping. The cells were transported from KHCPC to the operating room in refrigerated boxes controlled at 2°C–8°C. Cells were placed in transparent vials and were determined at the time of shipment to be free of foreign material by visual inspection.

All surgical procedures were performed under local anesthesia. Cell injection was performed based on a previous study[10] (Figures 2A and 2B). Using a 1.6-mm incision at the corneal limbus, we removed degenerated corneal endothelial cells (CECs) and the abnormal extracellular matrix from the patient's Descemet's membrane within an 8-mm-diameter region in the central cornea, using a silicone needle. (Inami, Tokyo, JAPAN). After confirming the extent of corneal endothelium removal by Vision Blue staining, the anterior chamber was washed with BSS. The incision was closed using 10-0 nylon sutures. Following preparation of the thawed cell stock in a syringe, 8 × 10^5 cells/160 μL of the cell suspension was injected into the anterior chamber. To promote adhesion of the injected cells, patients were positioned in a prone posture for a duration of 3 h immediately following the procedure. Following cell injection, the patient initiated a regimen of topical glucocorticoids (0.1% betamethasone, Rohto Nitten, Aichi, Japan) administered five times daily for the duration of the study. This was aimed at suppressing acute inflammation or immunologic reactions. Additionally, as per our standard protocol for corneal transplantation procedures, an antimicrobial eye drop (1.5% levofloxacin, Rhoto Nitten, Aichi, Japan) was used five times daily to prevent infection.

Primary and secondary outcomes

As our primary outcome measure, we evaluated all adverse events, including systemic symptoms. To assess adverse events associated with iPS cell-derived corneal endothelial substitute cells, we examined the following.

a) Unintended cell proliferation or the presence of a cell mass in the vitreous cavity.
b) Intraocular pressure elevation (defined as reaching 25 mmHg or more, with insufficient response to anti-glaucoma therapy for rapid pressure reduction).
c) Rejection, clinically characterized by a keratic precipitate score of 2 or 3 and an increase in corneal thickness compared to the previous observation point (However, if corneal thickness improves through adjustments such as increasing the dose of steroid eye drops, it is classified as suspected rejection). To evaluate adverse events resulting from surgery and procedures, we examined the following:
a) Presence of infection.
b) Progression of cataracts.
c) Occurrence of expulsive hemorrhage during or after surgery.

Visual acuity and corneal thickness were evaluated as secondary outcome measures.[10] The effectiveness of the treatment in improving visual acuity was determined based on specific criteria using the Landolt C eye chart (decimal visual acuity measurement). The key criterion for effectiveness was an improvement of at least two lines on the Landolt chart compared to baseline (before surgery). The decimal visual acuity was converted to LogMAR visual acuity to facilitate analysis. The evaluation criteria were as follows.

Visual acuity improvement

- **Effective**: Demonstrates improvement in visual acuity at least once before Visit 9, with sustained improvement in at least 2 visits, including the final evaluation.
- **Partially Effective**: Shows improvement in visual acuity at least once before Visit 9 but fails to maintain improvement at the final evaluation.
- **Ineffective**: Does not meet the criteria for either Effective or Partially Effective.

Corneal thickness improvement

- **Effective**: Demonstrates improvement in central corneal thickness (μm) by decreasing 20% or more from the preoperative measurement at each visit, with sustained improvement in at least 2 visits, including the final evaluation.
- **Partially Effective**: Shows improvement in corneal thickness at least once before Visit 9 but fails to maintain improvement at the final evaluation.
- **Ineffective**: Does not meet the criteria for either Effective or Partially Effective.

Overall treatment effectiveness

- If there is a decrease in visual acuity from baseline (preoperative visual acuity) and an increase in postoperative central corneal thickness compared to baseline (preoperative central corneal thickness) observed following Visit 9, the treatment is deemed 'Ineffective'.

Genome mutation analysis

Genomic mutations (SNVs/Indels and CNVs) were evaluated by whole genome sequence analysis and SNP array analysis. Briefly, whole genome sequencing library was generated using KAPA Hyper Prep Kit (Kapa Biosystems) without PCR and sequenced with NovaSeq6000 (Illumina) according to the manufactures' protocols followed by generating FASTQ files using bcl2fastq v2.20.0.422. SNP-genotyping arrays Infinium OmniExpress-24 v1.3 Kit (Illumina) experiment was performed, and a final report was exported using GenomeStudio 2.0.4 (Illumina). *De novo* genomic mutations in CLS001 was called as described previously[24] except that adapter trimming was performed with fastp.[19] Detected mutations were determined whether they were on tumor-related genes compiled in the Cancer Gene Census[15] and Shibata's list (http://www.pmda.go.jp/files/000152599.pdf).

Analysis of *EP300* mutation

After cell administration to the subject, the results of the final whole genome analysis revealed a deletion on the *EP300* gene. We then examined whether the mutation was homozygous or heterozygous, at what stage of production the cells with the mutation were contaminated, and what proportion of the CLS001 was contaminated with cells carrying the mutation.

Individual vials of MCB from the same lot used in this clinical study were thawed, cultured and clonally expanded, from which 20 colonies were chosen for the EP300 mutation. cDNA was constructed from each colony. Colony pickup yielded two types of iPS cells, one without the *EP300* mutation and the other with the mutation. For RT-PCR analysis, primers were designed to flank the deletion site (Table S8A). RT-PCR analysis was performed on cDNA from each colony using these primers to determine if the mutation was heterozygous or homozygous based on the mode of expression of the 303 base pair (bp) band of wild type (WT) *EP300* and the 225 bp band of the mutant *EP300*. Next, primers for quantitative RT-PCR were designed to span the border of the *EP300* deletion, and endogenous control primers were designed for the normal region away from the *EP300* deletion site (Table S8B). The cDNA was prepared by spiking the iPS cells with mutation at 0.01, 0.1, 1, 10, and 100% contamination rates to iPS cells without mutation. Quantitative RT-PCR was performed on each cell using the two primers, and a calibration curve was generated by calculating relative expression levels using the ΔΔCT method. Using this calibration curve, the mutant cell contamination rate of each lot of cells was measured by quantitative RT-PCR.

Screening of cell surface markers of CLS001 by flow cytometry analysis

Thawed CLS001 was analyzed for Screening of cell surface markers using BD Lyoplate Human Cell Surface Marker Screening Panel (BD Biosciences) at Kamakura Techno-Science Inc. (Kamakura, Japan). This material contains an array of antibodies specific for 242 different cell surface markers in three 96-well plates. Three lots of CLS001 were used for this screening and staining was conducted according to the manufacturer's procedures. Briefly, lyophilized antibody was reconstituted with 110 μL of phosphate-buffered saline (PBS)(−). Cell suspension of CLS001 was dispensed into three 96-well plates at 5-6 × 10^5 cells/well, and 20 μL of reconstituted antibody was added to the cells and incubated on ice for 20 min. Cells were then washed twice with ethylene diamine tetra-acetic acid (EDTA) + Stain Buffer and centrifuged at 300 × g for 5 min to remove unlabeled antibody. Secondary antibody was added at 100 μL/well and incubated on ice under light-shielded condition for 20 min. Cells were then washed twice with EDTA + Stain Buffer and centrifuged at 300 × g for 5 min. Then, 150 μL of EDTA + Stain Buffer was added, and an appropriate amount of 7-amino-actinomycin D (7-AAD) was added just before flow cytometry (FCM) measurement to stain dead cells.

BD LSRFortessaTM X-20 (BD Bioscience) was used for FCM, and 10,000 events of cells were captured in the gate per well. BD FACSDiva software Ver. 8.0.1 (Becton, Dickinson and Company) was used for analysis.

Undifferentiated iPS cell rate by OCT4 quantitative PCR

The method for measuring undifferentiated cell residuals and the resulting calibration curve are based on previous report.[12] Briefly QHJI01s04 iPS cells for preclinical study were mixed (spiked) in B4G12 cells in indicated concentrations (Figure S2) and analyzed for the expression level of *OCT4*. Total RNA was isolated from cells with the RNeasy kit (Qiagen). Isolated RNA was adjusted to 2 μg, and cDNA was synthesized using the ReverTra Ace α kit (Toyobo, Tokyo, Japan). Quantitative real-time PCR analysis was performed by SYBR Green enzyme mixture (Thunderbird SYBR qPCR mix; TOYOBO) and Step One Plus thermal cycler (Applied Biosystems, Foster City, CA). Primer pairs are listed in the following.

Gene	–	Sequence
POU5F1(OCT4)	Forward	GAAACCCACACTGCAGCAGA
POU5F1(OCT4)	Reverse	TCGCTTGCCCTTCTGGCG

Data analysis was performed using the Step One software, version 2.3 (Applied Biosystems). Expression level of each sample was measured by the average of dual experiments, and mean expression levels were presented as average ±SD of independent 3 lots. Mean expression levels were compared to B4G12 levels by the delta-delta CT method. This iPS mixing rate-*OCT4* expression rate curve was used as a calibration curve to quantitate residual undifferentiated iPS cells rate of CLS001. The red line in the calibration curve figure indicates that the undifferentiated cell residual rate is 0.01%.

Detection of residual undifferentiated iPS cells in CLS001 by expansion culture

CLS001 cells were disseminated on seven wells of iMatrix-511 (0.6 μg/cm^2)-coated 6-well culture plates in a 37°C, 5% CO_2 tissue-culture incubator, at a concentration 1 × 10^5 cells/well in Stemfit AK03N culture medium supplemented with 10 μM ROCK inhibitor Y27632 (Nacalai Tesuque, Kyoto, Japan). CLS001 cells spiked with iPS cells at 0.1% were also disseminated and cultured in the same manner as positive control. Culture medium was changed to AK03N without Y27632 on the following day. After 7 days culture, the cells were fixed and performed OCT4 immunostaining.

Karyotyping analysis

Karyotyping analysis was performed by G-banding methods using CLS001 at Chromocenter (now Sumika Chemical Analysis Service, Ltd., Osaka, Japan). Briefly, frozen stock of CLS001 was thawed and disseminated on iMatrix-511MG coated culture dishes with Corneal Endothelial Differentiation Medium containing Y27632. The medium was then changed with Corneal Endothelial Differentiation Medium without Y27632 every day, and Carnois-fixed and subjected to image analysis of mid-mitotic images after trypsin-Gimsa staining. Twenty metaphase cells were used for chromosome number and 8 metaphase cells were used for karyotype. If two or more of the same structural abnormality is observed, or two or more of the same chromosome increase is observed, or three or more of the same chromosome decrease is observed, they were judged to be clonal abnormalities.

Cell growth analysis

Cell growth analysis of CECSi cells was outsourced to CMIC Pharma Science Co., Ltd. (Yamanashi, Japan) using a protocol reported previously.[25] Briefly, frozen stocks of CLS001 WCB cells were thawed, and 1 × 10^6 cells were disseminated on T75 cell culture flasks. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (50 U/ml penicillin and 50 mg/mL streptomycin) and passaged every week until p = 11. Every passage harvested cell number was counted by automated cell counter.

Analysis of *EP300* gene mutation

The analysis of the entire genome, using cells from the same batch injected into the patient, uncovered a deletion in the *EP300* gene, which is documented in the Cancer Gene Census.[15]

Experiment design for iPSC analysis

We thawed three vials of research-grade iPSCs at passage 17, derived from the identical QHJI01s04 iPSC line utilized in the clinical study detailed in this manuscript, and cultivated them until passage 19 (Figure S9A). Subsequently, we extracted DNA and RNA from iPSCs at passage 19 and assessed the variant allele frequencies associated with the *EP300* deletion, as well as the disparities in RNA expressions. In addition, we examined the differentiation potential of iPSCs at passage 19 across all three germ layers.

iPSC culture

Three frozen vials (15M48, 15M49, 15M50) of iPSCs were thawed using a 37-degree Celsius water bath. After thawing, iPSCs were cultured using iMatrix-511MG (Matrixome) and Stem Fit AK03N (Ajinomoto) according to the method published previously.[24] During culture from passage 18 to passage 19, samples were prepared in triplicate (Figure S8A). At passaging 19, DNA and RNA were extracted using DNeasy Blood & Tissue Kit (QIAGEN) and RNeasy Mini Kit (QIAGEN) following to the manufacturers' protocols, and remaining cells were provided for differentiation analysis.

Droplet digital PCR analysis

To determine the frequency of the wild-type or mutant (delta chr22:41,559,981-41,560,191) allele in *EP300* of the established iPSC clones, droplet digital PCR (ddPCR) was performed with negative (no template) and positive (a mutant DNA fragment synthesized by IDT) controls. The PCR mixture in a total volume of 20 μL consisting of 1x ddPCR Supermix (no dUTP), Primer mix (1 μM), Probe mix (0.5 μM), gDNA (30 ng), HindIII (5 U) and water were mixed with droplet generator oil to make the droplets in a DG8 cartridge by a QX200 droplet generator (Bio-Rad). The droplets were then transferred gently into a 96-well plate for amplification by PCR with a C1000 Touch thermal cycler (Bio-Rad) according to the following protocol: 10 min at 95°C, followed by 40 cycles of 30 s at 94°C and 1 min at 56°C, followed by 10 min at 98°C; all ramp rates were 2°C/s. Primers and Probes were custom-made by IDT as follows: the forward primer is 5′-CCTGTACAACAGAGACCCTATC-3′, the reverse primer is 5′-AGATAGTAACTTTGAAGATATAATACAGTCA-3′, the probe for the wild-type allele is 5'-/56-FAM/TGTACAGAG/ZEN/TGCGGAAGAAAGATGCA/3IABkFQ-3′, the probe for the mutant allele is 5'-/5HEX/TCTTTGTCA+G+G+TTT+TTT+AT + TCT/3IABkFQ-3' (a base preceded by + is Locked Nucleic Acid). After the PCR reaction, the 96-well plate was incubated at a room temperature for 10 min, followed by each generated droplets were individually detected for specific fluorescence (FAM for the wild-type and HEX for the mutant) and analyzed by a QX200 droplet reader

with QuantaSoft version 1.7.4 (Bio-Rad). In the analysis, the number of positive and negative droplets for both fluorophores were measured with a manual threshold and then their ratio is adjusted to a Poisson distribution to determine the frequency of the mutant allele in samples.

Differentiation analysis

The trilineage differentiation capacity of iPSCs at passage 19 was confirmed using the STEMdiff Trilineage Differentiation Kit (#05230, STEMCELL TECHNOLOGIES) according to the manufacturers' instructions. Cells were harvested and fixed for the analysis of lineage-specific markers on day 7 for ectoderm and day 5 for mesoderm and endoderm lineages. Subsequently, they were assessed using flow cytometry with specific antibodies (Nestin and Pax6 for ectoderm, Brachyury (T) and Ncam for mesoderm, and Sox17 and Foxa2 for endoderm).

RNA-sequencing analysis

Stranded Illumina libraries were prepared using TruSeq Stranded Total RNA Library Prep Kit Gold. The libraries were sequenced with Illumina NovaSeq 6000 with 2 × 101 cycles. All the experiments were conducted according to the manufacturers' instructions. Raw sequenced files were demultiplexed and converted into FASTQ files using bcl2fastq v2.20.0.422. Gene expression matrix was obtained using the pipeline developed in ENCODE project (*https://www.encodeproject.org/pipelines/ENCPL002LPE/*), where Homo_sapiens.GRCh38.dna.primary_assembly.fa in GRCh38 ENSEMBL release 109 and gencode.v43.primary_assembly.annotation.gtf in GRCh38 GENCODE release 43 were used. The expected gene count matrix calculated with RSEM was further analyzed with DESeq2 v1.38.3[20] on R v4.2.3(R Core Team (2023). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) and differentially expressed genes were identified with Adjusted p-value <0.01. With differentially expressed gene sets, gene ontology and the pathway analysis were performed by Enrichr.[21–23]

QUANTIFICATION AND STATISTICAL ANALYSIS

The maximum enrollment in this study was 3 patients. The study was originally planned for a statistical analysis of each endpoint. However, as this study included only one patient due to reasons described in the Results section, statistical analysis was not performed.

ADDITIONAL RESOURCES

Clinical study registry numbers and links: jRCTa031210199
https://jrct.niph.go.jp/en-latest-detail/jRCTa031210199.