1 Statistical Analyses

2 Values are presented as means ± standard deviations. Differences between the groups were

3 analyzed with unpaired Student's *t* tests. All *t* tests were two-side, and a *P*-value of <0.05 was

4 considered to be statistically significant.

5

6 **RESULTS**

7 Comparison of mRNA Expression of BTB/POZ-Zinc finger-containing Transcription Factors in

8 Confluent and Subconfluent Cultured HCECs in vitro and Normal HCECs in vivo

9 The expression of six human BTB/POZ-Zinc finger-containing transcription factors,

10 promyelocytic leukemia zinc finger (PLZF), B-cell lymphoma 6 (BCL-6), Kaiso, myoneurin,

11 KIAA0441, and zinc finger protein278 (ZNF278), were compared in confluent and subconfluent

12 cultured HCECs by RT-PCR because the gene with a different expression pattern was considered

13 to be a good candidate for regulating the proliferation of HCECs. The results showed that the

14 mRNA of PLZF was expressed only when HCECs were confluent and the expression of the

15 mRNA of the other transcription factors was unchanged in both confluent and subconfluent

16 HCECs. The mRNA of PLZF was also undetectable by RT-PCR up to 40 cycles in

17 subconfluent cultured HCECs (Fig. 1A). These results were consistent with the findings in all

18 HCECs from different human donors. The mRNA of PLZF was also found to be expressed in

1 the corneal endothelial cells obtained from normal human corneas (ages: 5, 59, 62, 67, and 73

2 years; Fig. 1B).

3

4 Kinetics of PLZF mRNA Expression in Primary Cultured HCEC:

5 Next, the kinetics of the PLZF mRNA expression in primary cultured HCECs was examined by

6 real-time PCR. The mRNA of PLZF was not expressed when HCECs were still in the

7 proliferation phase, but began to be expressed at the 100% confluency (1 day) which seemed to

8 occur when they reached confluency (Figure 2B). The mRNA of PLZF was undetectable by

9 RT-PCR up to 40 cycles in cultured HCECs of 40%, 60%, and 80% confluency (Figure 2C).

10 After reaching confluency, the relative expression level of PLZF mRNA continued to increase

11 for up to 5 days (Figs. 2B, 2C). Cell-cell contacts were still not formed in the proliferation

12 phase, but were formed after the 100% confluency (1 day) phase as was seen in cells in the

13 non-proliferation phase (Figs. 2A). These results led us to hypothesize that the PLZF

14 expression might be related to cell-cell contact and should be investigated in more detail.

15

16 Effect of Disruption of the Cell-Cell Contact on PLZF mRNA Expression:

17 To determine whether cell-cell contact is associated with the expression of the *PLZF* gene,

18 confluently-cultured HCECs were incubated with 3.2 mM EDTA for 2 h and replaced with

1 normal culture medium containing no EDTA (Fig. 3A). After the treatment, HCECs were
2 harvested at specific times, and the changes of the mRNA of PLZF were assessed by real-time
3 PCR. The results showed that the expression of the mRNA of PLZF decreased by about
4 20-fold at 3 h after the EDTA treatment, but began to increase at 24 h after replacing the EDTA
5 media with normal media. The level of PLZF recovered to the original level when the cell-cell
6 contacts were reformed at 72 h (Fig. 3B).
7 When the same experiment was performed on cultured human umbilical vein endothelial cells
8 (HUVEC), the expression of PLZF mRNA was not changed by EDTA treatment (Fig. 3C),
9 suggesting the importance of PLZF in the cell-cell contact of corneal endothelial cells.
10

11 Effect of PLZF Gene Transfer on the Proliferation of Cultured HCECs

12 To test the hypothesis that the PLZF may suppress the proliferation of HCECs, we first infected
13 subconfluent cultured HCECs with Ad-PLZF at an MOI of 50, 100, and 200. The efficiency of
14 infection of the Ad-GFP vectors into HCECs at 48 h was 14% at an MOI of 50, 24% at an MOI
15 of 100, and 41% at an MOI of 200 (Fig. 4A). At the same time, Western blot analysis using
16 anti-PLZF antibody revealed a single band of approximately 70 kDa. The intensity of the bands
17 was strongly correlated to the multiplicity of infection (Fig. 4B). Because PLZF mRNA was
18 expressed at low levels in the uninfected cultured HCECs, PLZF protein may be undetectable by

1 the Western blot analysis employed in this study.

2 The cell proliferation assay was done using the RT-CES system with the Ad-LacZ cells

3 serving as the control. When the cell proliferation rate was continuously monitored, it was

4 found that infection with Ad-PLZF at an MOI of 100 inhibited the cell growth by 30.3% at 24

5 hours and 26.5% at 48 hours compared to that with Ad-LacZ (Fig. 4D). This inhibition was

6 statistically significant, and the effect lasted up to 72 h (Fig. 4C).

7

8 Comparison of mRNA Expression of Cadherin Family in Cultured HCECs in vitro and Normal

9 HCECs in vivo, and Kinetics of N-Cadherin mRNA Expression in Primary Cultured HCEC

10 The expression of five members of the human cadherin family, E-cadherin, N-cadherin,

11 VE-cadherin, V-cadherin, and P-cadherin, were compared in vitro HCECs, in vivo HCECs, in

12 vitro HUVEC, and normal human skin by RT-PCR. N-cadhein and V-cadherin were detected in

13 in vitro HCECs, whereas only N-cadherin was detected in in vivo HCECs (Fig. 5A).

14 Next, the kinetics of the expression of the mRNA of the N-cadherin was examined in primary

15 cultured HCECs by real-time PCR. In contrast to the expression of PLZF, N-cadherin

16 expression was detected at high levels in subconfluent HCECs, and decreased as cultured

17 HCECs attained confluency (Fig. 5B). When the same experiment was performed on the

18 mRNA expression of ZO-1, no change was found in the kinetics of ZO-1 mRNA expression (Fig.

1 5C).

2

3 Determination of PLZF-induced Gene Expression in HCECs

4 Finally, the changes in the expression of genes in HCECs induced by PLZF were determined by
5 DNA microarray analysis. When a total of 30,000 genes were analyzed, PLZF was shown to
6 up-regulate at least 54 genes and down-regulate at least 34 genes. Unexpectedly, the expression
7 of *cyclin A2* and *c-myc* genes were not affected by an over-expression of the PLZF gene in CECs
8 (data not shown). Two growth factors including heparin-binding epidermal growth factor-like
9 growth factor (HB-EGF) and connective tissue growth factor were the most down-regulated by
10 0.059-fold and 0.188-fold respectively. Of particular interest was the discovery that the
11 transforming growth factor-β stimulated clone 22 *(TSC-22)* gene was up-regulated by 2.32-fold
12 (data not shown). The increased expression of the mRNA of TSC-22 was also confirmed by
13 real-time PCR (Fig. 6).

14

15 Expression of PLZF mRNA in Corneal Endothelial Cells with Iridocorneal Endothelial
16 Syndrome (ICE syndrome)

17 We have examined the expression of the mRNA of PLZF in corneal endothelial cells obtained
18 from three patients with the ICE syndrome (Fig.7A), and found that the relative expression of the

1 mRNA of PLZF was lower in these patients than in normal controls by real-time PCR (Fig.7B).
2 In particular, the expression of PLZF mRNA was undetectable in Chandler's syndrome (Fig.7B,
3 7C). The relative expression of the mRNA of PLZF as normal controls is average value of four
4 normal human corneal endothelial cells (ages; 62, 67, 73, and 73 years).
5

6 **DISCUSSION**

7 PLZF is a transcriptional repressor and is known to suppress the expression of several genes
8 which regulate cell proliferation. Thus in earlier studies, an enhanced expression of PLZF led
9 to the suppression of proliferation in some cell lines [11, 21]. In murine 32Dcl3 cells, the cell
10 cycling profiles of over-expressing PLZF were significantly altered, and up to 80% of the cells
11 accumulated in the G0/G1 phase with a significantly smaller proportion of cells than in the S
12 phase [21].
13 Our results showed that, among the different members of the BTB/POZ-zinc finger family,
14 PLZF was the only gene which varied in association with the alterations of HCECs. Thus,
15 PLZF was not expressed in HCECs in the proliferative phase and was later expressed when the
16 cultured cells reached confluency. We also found that the expression of the PLZF mRNA was
17 profoundly decreased when the cell-cell contact was disrupted by EDTA treatment and returned
18 to the original level as the cell-cell contact was reformed. These changes in the expression

1 pattern suggest that PLZF gene expression may be regulated by cell-cell contact and related to
2 the proliferation of HCECs. In fact, our results are quite consistent with recent reports in which
3 EDTA-exposed corneal endothelial cells were released from contact inhibition and proliferated
4 [22]. Another study has shown that the cell-cell contact-induced inhibition is mediated, at least
5 in part, by $p27^{kip1}$ because the $p27^{kip1}$ protein level is 20 times higher in confluent rat corneal
6 endothelial cells than in subconfluent cells. In addition, the level of the $p27^{kip1}$ protein is
7 substantially lower in EDTA-treated confluent cells than in untreated control cells [23].
8 Although the link between PLZF and $p27^{kip1}$ has not been determined, both genes presumably
9 contribute to the contact inhibition of HCECs.

10 The cell adhesion molecules expressed in corneal endothelial cells are ZO-1, connexin-43, and
11 cadherin [24-26]. Among these, the cadherins are major intercellular adhesion molecules that
12 mediate calcium-dependent cell-cell adhesion through homophilic interactions [27]. It has been
13 demonstrated that a breakdown in the cadherin-mediated cell adhesion activates a
14 ß-catenin-mediated intracellular signaling pathway, inducing the expression of a set of genes,
15 including cyclin D1, c-myc, and c-jun, that are critical for cell proliferation and cell survival
16 [28-30].

17 We have examined the expression of cadherin family mRNA in cultured HCECs and in vivo
18 HCECs, and N-cadherin was found to be the major cadherin in HCECs. In addition, the

expression of N-cadherin increased in proliferating HCECs before the expression of PLZF (Figure 5B). We have hypothesized that N-cadherin and its downstream signaling pathways are the candidate molecules involved in the regulation of the expression of the *PLZF* gene. Currently, experiments are being carried out in our laboratory to test this hypothesis.

The involvement of PLZF in suppressing the proliferation of cultured HCECs has been clearly demonstrated by infection of the *PLZF* gene in HCECs. The degree of the suppression was not as high as in murine 32Dcl3 cells, but considering an infection efficiency of 24% at an MOI of 100 with the Ad-GFP vector, an over-expression of PLZF led to a relatively high degree of suppression of the proliferation of HCECs. In addition, TSC-22 was found to be increased in HCECs which over-expressed the *PLZF* gene according to the DNA microarray analysis. TSC-22, a leucine zipper transcriptional factor, was found to be an immediate-early target gene of TGF-beta 1 [31] and has the characteristics of a suppressor of cell proliferation [32-34]. Moreover, TSC-22 binds to and modulates the transcriptional activity of Smad3 and Smad4, and it enhances TGF-β signaling by associating with Smad4 [35]. Evidence has been showing that TGF-β_2 is present in high concentrations in normal aqueous humor [36, 37], and it suppresses the proliferation of rabbit and rat corneal endothelial cells in vitro [7, 38, 39]. Thus, *TSC-22* might be involved as a target gene for PLZF in suppressing HCEC proliferation in accordance with TGF-β signaling pathway. This hypothesis, however, needs further investigation.

We have also examined the expression of PLZF mRNA in the corneal endothelial cells obtained from three patients with iridocorneal endothelial syndrome (ICE syndrome). Interestingly, the relative expression of the mRNA of PLZF in ICE syndrome was lower than in normal controls by real-time PCR. In particular, the expression of PLZF mRNA was undetectable in Chandler's syndrome (Figure 7). These findings suggest that the absence of the *PLZF* gene may lead to the abnormal proliferation of corneal endothelial cells, a hallmark of the ICE syndrome.

In conclusion, we have shown that the mRNA of PLZF was closely associated with cell-cell contact phenomenon of human corneal endothelial cells. Further studies are needed to clarify the correlation of the *PLZF* gene with the intracellular signals governed by TGF-beta and with the expression of the cell adhesion molecule such as cadherin. As PLZF is normally expressed in the human corneal endothelium, it can serve as a possible target to modulate cell proliferation. It may be possible in the future to treat patients with severe corneal endothelial damage if the key factor regulating the expression of the *PLZF* gene and the genes regulated by PLZF are identified.

Acknowledgments:

The authors thank Y. Hanakawa and T. Tsuda for the construction of the adenovirus vector.

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16

17

18

1 **Figure legends**

2 **Figure 1.** Expression of the mRNA of BTB/POZ-zinc finger-containing transcription factors

3 by RT-PCR.

4 A. Ethidium-bromide-stained agarose gels showing PCR products for PLZF, BCL-6, Kaiso,

5 myoneurin, KIAA0441, ZNF278 and GAPDH. Similar results were obtained in two other

6 experiments. (35 cycles)

7 Lane 1: Sub-confluent cultured HCECs; Lane 2: 100% confluent cultured HCECs; Lane 3: in

8 vivo normal HCECs.

9 B. Ethidium-bromide-stained agarose gels showing PCR products for PLZF in vivo normal

10 human corneas, ages: 5, 59, 62, 67, and 73 years (40 cycles).

11

12 **Figure 2.** Kinetics of mRNA of PLZF in primary cultured HCECs.

13 A. Cell number in 6 well plates for each condition.

14 B. Time course of the expression of the PLZF mRNA in cultured HCECs.

15 Cultured HCECs were collected at 40%, 60%, 80% and 100% (1 day, 3 days, 5 days, and 7 days

16 after reached confluency). The relative expression of PLZF mRNA was determined by

17 real-time PCR, and the amount of each mRNA was calculated relative to the amount of GAPDH

18 mRNA in the same sample (n = 3 each). The ratio of the sample from 100%/1 day was set to

1 “1”).

2 C. Ethidium-bromide-stained agarose gels showing PCR (40 cycles) products for PLZF and

3 GAPDH.

4

5 **Figure 3.** Effect of disruption of the cell-cell contact on PLZF mRNA expression.

6 A. Effect of 3.2 mM of EDTA on the integrity of human corneal endothelial cell-cell contact.

7 1: No treatment: cell-cell contact is intact.

8 2: Incubation with EDTA for 2 h leads to moderate cell-cell separation.

9 3: Incubation in medium without EDTA for 24 h after incubation with EDTA for 2 h. Cell-cell

10 contact has recovered.

11 B. HCECs were cultured until confluent, and then the medium was replaced by medium

12 containing 3.2 mM EDTA for 2 h and returned to the normal culture medium for 72 h. The

13 relative expression of PLZF mRNA was determined by real-time PCR and the amount of mRNA

14 was calculated relative to the amount of GAPDH mRNA in the same sample. The ratio of the

15 sample from 3 h post-incubation was set to 1. This experiment was repeated twice.

16 C. The same experiment was performed on cultured human umbilical vein endothelial cells.

17 The ratio of the sample from subconfluent culture was set to 1.

18

1 **Figure 4.** Efficiency of adenovirus vector infection and effect of PLZF on proliferation of

2 cultured HCECs.

3 A. GFP-positive cells in cultured HCECs 48 h after transfection. HCECs at 80% confluency

4 were infected at an MOI of 50, 100, and 200 with an adenovirus vector expressing GFP.

5 Phase-contrast photograph (left) and fluorescence photograph (right) showing the infection

6 efficiency was 14% at an MOI of 50, 24% at n MOI of 100, and 41% at an MOI of 200.

7 B. Western blot analysis with anti-PLZF antibody of cultured HCECs infected with Ad-PLZF

8 and uninfected cells. HCECs at 80% confluency were infected with Ad-PLZF. The cells were

9 harvested 48 h after infection. Lane 1: MOI 50; Lane 2: MOI 100; Lane 3: MOI 200; Lane 4:

10 uninfected HCECs.

11 C. The cells were harvested at 24 h after infection, and the cells were seeded into a 16-well

12 strip. Cell index values were determined every hour automatically by the RT-CES system for

13 up to 72 h. Each blot is an average of 8 samples.

14 D. Cell index values at 24 and 48 h are shown. Error bars are standard deviations.

15 *Significant difference (P <0.001) between Ad-PLZF and Ad-LacZ (n = 8 each).

16

17 **Figure 5.** The expression of mRNA of cadherin family and ZO-1.

18 A. Ethidium-bromide-stained agarose gels showing PCR products for cadherin family.

1 (40 cycles)

2 1: In vitro HCECs, 2: in vivo HCECs, 3: in vitro HUVEC, 4: normal human skin.

3 B,C. Time course of the expression of the N-cadherin and ZO-1 mRNA in cultured HCECs.

4 Cultured HCECs were collected at 40%, 60%, 80%, and 100% (1 day, 3 days, 5 days, and 7 days,

5 respectively) after reached confluency. The relative expressions of N-cadherin and ZO-1

6 mRNA were determined by real-time PCR, and the amount of each mRNA was calculated

7 relative to the amount of GAPDH mRNA in the same sample (n = 3 each). The ratio of the

8 sample from 100%/5day was set to 1 for N-cadherin and 100%/3day to 1 for ZO-1.

9

10 **Figure 6.** The expression of the TSC-22 mRNA in cultured HCECs.

11 HCECs at 50% confluency were infected with Ad-PLZF or Ad-LacZ at an MOI of 100. The

12 cells were harvested 48 h after infection. The relative expression of TSC-22 mRNA was

13 determined by real-time PCR, and the amount of each mRNA was calculated relative to the

14 amount of GAPDH mRNA in the same samples. The ratio of the sample from Ad-LacZ was set

15 to 1). *Significant difference (P <0.005) between Ad-PLZF and Ad-LacZ (n = 4 each).

16

17 **Figure 7.** Expression of the mRNA of PLZF in the corneal endothelial cells from patients with

18 the ICE syndrome.

1 A. 1: Chandler's syndrome, 2: Progressive essential iris atrophy, 3: Cogan-Reese syndrome.

2 B. The relative expression of PLZF mRNA was determined by real-time PCR, and the amount of

3 each mRNA was calculated relative to the amount of GAPDH mRNA in the same samples. The

4 ratio of the sample from progressive essential iris atrophy was set to 1).

5 C. Ethidium-bromide-stained agarose gels showing PCR products for PLZF. (40 cycles)

6

7