(unpublished data). In addition, the kinetics of TSC-22 mRNA were very similar to that of TGF-β_2 mRNA when cultured HCECs, thus the expressions of both TSC-22 and TGF-β_2 mRNA were increased in a cell density-dependent manner as with the kinetics of PLZF mRNA (unpublished data). Our results together with previous findings suggest the hypothesis that TSC-22 may be involved in the suppression of proliferation of HCECs binding to the Smad complex to enhance TGF-β signaling as a downstream gene of PLZF.

In conclusion, PLZF probably play a key role in the suppression of proliferation of HCECs, and PLZF may be one of the targets for regenerative medicine in treating the patient with corneal endothelial diseases.

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Table **Expression profile obtained by array hybridization**

Genes[a]	Description	Accession number	Ratio (*PLZF/LacZ*)
znf145	Promyelocytic leukemia zinc finger protein	NM_006006	334.752
znf226	zinc finger protein 226	NM_016444	2.470
tsc-22	transforming growth factor beta-stimulated protein tsc-22	NM_006022	2.323
dtr	heparin-binding epidermal growth factor-like growth factor	NM_001945	0.059
ctgf	connective tissue growth factor	NM_001901	0.188
ptgs	prostaglandin-endoperoxide synthase2	NM_000963	0.219
ngfb	nerve growth factor, beta polypeptide	NM_002506	0.352

Figure legends

Figure 1. Expression pattern of mRNA of transcriptional repressors. Ethidium-bromide-stained agarose gels showing PCR products for PLZF, BCL-6, Kaiso, myoneurin, KIAA0441, ZNF278 and GAPDH.

Lane 1, Sub-confluent cultured HCECs; lane 2, 100% confluent cultured HCECs; lane 3, in vivo normal HCECs.

Figure 2. Kinetics of mRNA of PLZF.

A. Time course of PLZF mRNA expression in cultured HCECs. Cultured HCECs were collected at 40%, 60%, 80%, and 100% (2 days, 4 days, 6 days, and 8 days) confluency. The relative expression of PLZF mRNA was determined by real-time quantitative PCR and the amount of each mRNA was calculated relative to the amount of GAPDH mRNA in the same samples (n = 3 each).

B. Ethidium-bromide-stained agarose gels showing PCR products for PLZF and GAPDH.

Figure 3. Effect of PLZF on proliferation of cultured HCECs.

HCECs at 80% confluency were infected with Ad-PLZF or Ad-LacZ, and were harvested at 24 hours after infection.

A. Cell index values were determined every hour automatically by the RT-CES system for up to 72 hour. Error bars are standard deviations (n = 8 each).

B. Cell index values at 24 and 48 hour are shown. Error bars are standard deviations.

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

Figure 4. Effect of cell-cell contact on PLZF mRNA expression.

HCECs were cultured until confluent, and then the medium was replaced by medium containing 1.2 mg/ml EDTA for 120 minutes and returned to the normal culture medium for up to 72 hour. The relative expression of PLZF mRNA was determined by real-time quantitative PCR and the amount of each mRNA was calculated relative to the amount of GAPDH mRNA in the same samples.

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Effects of Promyelocytic Leukemia Zinc Finger Protein on the Proliferation of Cultured Human Corneal Endothelial Cells

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ABSTRACT

Purpose: To determine whether the promyelocytic leukemia zinc finger (PLZF) protein, a transcriptional repressor and negative regulator during cell cycling, plays a role in the proliferation of cultured human corneal endothelial cells (HCECs).

Methods: The expressions of the mRNA and the protein of PLZF were determined by real-time PCR and Western blot analysis, respectively. The changes in the expression of the PLZF gene of cultured HCECs were investigated at different times after the cell-cell contacts were disrupted by incubation with EDTA. The cell proliferation rate was assessed with a real-time cell electronic sensing (RT-CES) system after cultured HCECs were infected with either PLZF or LacZ encoding adenovirus vector (Ad-PLZF or Ad-LacZ) The PLZF-regulating genes were analyzed by DNA microarray analysis in cultured HCECs infected with Ad-PLZF.

Results. The expression of the mRNA of PLZF was first detected when the cultured HCECs became confluent, and the relative amount of PLZF mRNA continued to increase for up to 5 days as the cell-cell contacts were formed more firmly. On the other hand, the expression of the mRNA of PLZF decreased about 20-fold 3 h after EDTA exposure, and gradually returned to the original level as the cell-cell contacts were reformed at 72 h after the exposure. The assessment using the RT-CES system showed that the proliferation of cultured HCECs was inhibited for up to 72 h when infected by Ad-PLZF. DNA microarray analysis revealed that transforming

growth factor-β stimulated clone 22 *(TSC-22)* gene was up-regulated by 2.32-fold when infected by Ad-PLZF.

Conclusions: These findings indicate that the expression of PLZF in HCECs is closely related to the formation of cell-cell contacts, and that PLZF may play a role in suppressing their proliferation.

INTRODUCTION

The corneal endothelium is a single layer of cells lining the posterior surface of the cornea, and it helps maintains corneal transparency by regulating the hydration of the corneal stroma. It is widely accepted that corneal endothelial cells do not proliferate in humans once the endothelial monolayer is formed [1]. It is also known that the density of human corneal endothelial cells (HCECs) decreases by approximately 0.5% per year throughout life, and that the enlargement of the remaining cells compensates for this reduction to maintain corneal endothelial function [1-4]. In addition, a variety of injuries such as surgical stress during intraocular surgery, corneal trauma, and viral infections often cause an extensive reduction in the number of corneal endothelial cells, which can result in corneal endothelial dysfunction known as bullous keratopathy. Although penetrating keratoplasty (PKP) and other modern surgical procedures, including deep lamellar endothelial keratoplasty (DLEK), Descemet's stripping, and automated endothelial keratoplasty (DSAEK), can restore vision in such patients, it would be more beneficial to regulate the proliferation of corneal endothelial cells by manipulating cell cycles.

It has been demonstrated that HCECs are arrested in the G1 phase of the cell cycle in vivo rather than resting in the G0 phase [5]. In fact, HCECs have been shown to possess a strong potential to proliferate depending upon the age when they are cultured [6]. Among the anti-mitotic factors associated with the G1 arrest of the HCECs, TGF-β_2 is known to block the

G1-to-S transition by blocking the phosphorylation of $p27^{kip1}$, which is required for the nuclear export of the inhibitory molecules for degradation [7, 8]. However, the mechanisms involved in the cell cycling and proliferation of HCECs have not been fully determined as yet. In searching for a cell cycling mechanism, we focused on DNA binding transcriptional factors, including the members of the BTB/POZ-zinc finger protein family. The BTB/POZ-zinc finger proteins are sequence-specific transcriptional repressors characterized by a BTB/POZ domain, which is responsible for transcriptional repression, and a zinc finger domain that forms the DNA binding domain [9]. Among the family members, the *PLZF* gene was first identified by its fusion to the retinoid acid receptor (RAR) alpha locus in a therapy-resistant form of acute promyelocytic leukemia associated with the t(11;17) translocation [10]. *PLZF* is a sequence-specific DNA binding transcriptional repressor that suppresses the transcription of genes such as *cyclin A2* and *c-myc* [11-13]. In addition to these genes, it has been reported that pre-B-cell leukemia transcription factor 1 (Pbx1) can be a target gene for PLZF to suppress melanoma cell growth [14].

In preliminary experiments, we investigated the expression pattern of several members of the BTB/POZ-zinc finger protein family that were considered to be negative regulators in the cycling of HCECs. Interestingly, the expression level of the mRNA of PLZF alone was found to vary according to the state of the cell-cell contact. Thus, the purpose of this study was to

investigate the sequential changes in the expression of the mRNA of PLZF in HCECs in the primary culture and after EDTA exposure. In addition, we examined what role PLZF plays in the proliferation of HCECs using an adenovirus vector carrying genes encoding PLZF.

MATERIALS AND METHODS

Media and Culture Conditions

All primary and passaged HCECs were cultured in a media consisting of Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum, 30 mg/L of L-glutamine, 2.5 mg/L of Fungizone (GIBCO, Grand Island, NY), 2.5 mg/L of doxycycline (Sigma-Aldrich Co), and 2 ng/mL of basic fibroblast proliferation factor (Invitrogen, Carlsbad, CA) [15]. Cultured HCECs were maintained in a humidified incubator at 37° C and 10% CO_2.

Primary Cultures of HCECs

All procedures including those on human subjects were conducted in accordance with the principles of the Declaration of Helsinki [16], and this study was approved by Institutional Review Board of Ehime University.

Primary cultures of HCECs were started from normal human corneas acquired from the American Eye Bank. Human tissue was used in strict accordance with the tenets of the

Declaration of Helsinki. The corneoscleral buttons were stored in Optisol (Chiron, Irvine, CA) at 4° C and were cultured within 10 days of enucleation. Small explants from the endothelial layer, including Descemet's membrane, were removed with sterile surgical forceps and cultured in 35mm culture dishes coated with mouse collagen type IV (BD Biosciences, San Jose, CA). When a sufficient density of proliferating cells was attained, the cultured HCECs were rinsed three times in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(-)), trypsinized for 2 minutes at 37° C, and passaged at ratios ranging from 1:1 to 1:4, depending on the number of proliferating colonies. All subsequent passages were carried out using the same method, but at a ratio of 1:16. The approximate time to confluence after each passaging was 6 to 8 days [15]. We used cultured human corneal endothelial cells of the fifth passage for the experiments.

RNA Extraction and RT-PCR

Total RNA was isolated from HCECs and cultured HCECs using TRIzol reagent according to the manufacturer's instructions (Invitrogen). They were further purified by RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized with Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen).

PCR amplification was performed with TaKaRa Ex Taq (TaKaRa, Kusatsu, Japan) under the

following conditions: 94° C for 5 min, 35 cycles of denaturation at 94° C for 10 sec, annealing at 60° C for 20 sec, and extension at 72° C for 30 sec. The primer pairs used for RT-PCR are listed in Table 1 A.

Real-time PCR

Real-time PCR was performed with the DyNAmo STBR Green qPCR kit (FINNZYMES, Espoo, Finland) under the following conditions: 95° C for 15 min, 40 cycles of denaturation at 95° C for 10 sec, annealing at 60° C for 20 sec, and extension at 72° C for 30 sec by OPticon2 DNA Engine (BIO RAD, Hercules, CA). The primer pairs used for real time PCR are listed in Table 1B. The Ct values were determined by Opticon2 software, and the amount of each mRNA was calculated relative to the amount of GAPDH mRNA in the same samples [17]. Each run was completed with a melting curve analysis to confirm the specificity of the amplification and the absence of primer dimmers.

Releasing Model of Cell-Cell Contacts

To examine the effect of cell-cell contact on the expression of the *PLZF* gene, we first determined the effective concentration of EDTA that altered the integrity of endothelial cell-cell contact. Cultured HCECs were incubated for 2 h in 2.7, 3.2, and 4.0 mM of di-sodium

EDTA.2H_2O (EDTA). EDTA was prepared in DMEM with 15%FBS. Exposure to 2.7 mM of EDTA for 2 h caused a mild lateral separation of the cells, and incubation in 3.2 mM for 2 h caused a moderate lateral separation of the cells. Incubation in 4.0 mM of EDTA for 2 h caused a marked lateral separation and loss of contact to the dish. When 3.2 mM EDTA was used for 2 h to disrupt the cell-cell contacts of HCECs, the lateral separation of the cells was reversed by replacing the EDTA-rich media with normal culture medium. Therefore, we decided to use 3.2 mM EDTA for 2 h as the condition to disrupt the cell-cell contacts.

HCECs were cultured until confluent, and then the medium was replaced by one containing 3.2 mM EDTA. Cultured HCECs were treated with EDTA for 2 h and returned to the normal culture medium for up to 72 h. Cultured HCECs were collected at 1, 3, 6, 24, and 72 h after returning the cells to the normal culture medium, and the relative amounts of the mRNA of PLZF were evaluated by real-time PCR.

Adenovirus Vector Construction and Infection into Cultured HCECs

The degree of infection by GFP-expressing adenovirus vector (Ad-GFP) of the cultured HCECs was determined by counting the number of GFP positive cells when infected at a multiplicity of infection (MOI) of 50, 100, and 200. Adenovirus vector carrying genes encoding PLZF (Ad-PLZF) or LacZ (Ad-LacZ) were prepared using an adenovirus expression vector kit (Takara

Biomedicals) as described [14, 18]. Purified, concentrated, and titer-checked viruses were used for the infections.

Western Blot Analysis

HCECs in culture dishes were rinsed two times with PBS(-) and then lysed with Laemmli sample buffer (BIO-RAD) with β-mercaptoethanol, and the final concentration was 5%. Equivalent volumes of samples were separated on 7.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) and transferred to polyvinylidene (PVDF) membranes. After blocking with 5% nonfat dry milk and 0.1% Tween-20 in PBS, the membrane was incubated with monoclonal anti-human PLZF antibody (Oncogene Research Products, Cambridge, MA) for 1 h followed by specific secondary antibodies. The positive immunoreactions were made visible by an enhanced chemiluminescence (ECL plus) detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

In Vitro Cell Proliferation

The rate of cellular proliferation was analyzed with a real-time cell electronic sensing (RT-CES) system (ACEA Bioscience, San Diego, CA). Cells were grown on the surfaces of microelectronic sensors, which are composed of a circle-on-line electrode arrays and are

integrated into the bottom surfaces of the microtiter plate. Changes in cell number was monitored and quantified by detecting sensor electrical impedance. For cell quantification and viability measurements, the data generated on the RT-CES system correlated well with those from the colorimetric (MTT) assay. Cell index (CI) values obtained on the RT-CES system were quantitatively correlated with the cell numbers [19, 20].

The cells were harvested 24 h after infection with Ad-PLZF or Ad-LacZ at an MOI of 100 and seeded into a 16-well strip at a density of 1×10^4 cells/well. The sensor devices were placed into the 5% CO_2 incubator, and the cell index value was determined every hour automatically by the RT-CES system for up to 72 h.

Microarray Analyses

Cultured HCECs of 50% confluency were infected with Ad-PLZF or Ad-LacZ at an MOI of 100. Total RNA was isolated from Ad-PLZF or Ad-LacZ infected HCECs at 48 h post-infection. The Acegene Human oligo chip 30K (Hitachi Software Engineering, Japan) containing 30,000 genes was used to compare gene expression in cultured HCECs infected with Ad-PLZF or Ad-LacZ. Initial data analysis for each chip was performed using DNASIS-Array software (Hitachi Software Engineering, Japan).