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Research Paper

Restricted Presence of POU6F2 in Human Corneal Endothelial Cells Uncovered by Extension of the Promoter-level Expression Atlas

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ABSTRACT

Corneal endothelial cells (CECs) are essential for maintaining the clarity of the cornea. Because CECs have limited proliferative ability, interest is growing in their potentially therapeutic regeneration from pluripotent stem cells. However, the molecular mechanisms of human CEC differentiation remain largely unknown. To determine the key regulators of CEC characteristics, here we generated a comprehensive promoter-level expression profile of human CECs, using cap analysis of gene expression (CAGE) with a single molecule sequencer. Integration with the FANTOM5 promoter-level expression atlas, which includes transcriptome profiles of various human tissues and cells, enabled us to identify 45 promoters at 28 gene loci that are specifically expressed in CECs. We further discovered that the expression of transcription factor POU class 6 homeobox 2 (POU6F2) is restricted to CECs, and upregulated during human CEC differentiation, suggesting that POU6F2 is pivotal to terminal differentiation of CECs. These CEC-specific promoters would be useful for the assessment of fully differentiated CECs derived from pluripotent stem cells. These findings promote the development of corneal regenerative medicine.

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1. Introduction

Cornea is a transparent, avascular tissue located at the front of the eye. Corneal endothelium is the innermost monolayer of the cornea attached to Descemet's membrane. Corneal endothelial cells (CECs) play a crucial role in the maintenance of corneal transparency, by controlling the movement of ions and water between the corneal stroma and the anterior chamber (Hodson and Miller, 1976; Maurice, 1972). Because human CECs have limited proliferative ability (Joyce et al., 1996), significant loss of CECs due to disease or trauma can cause corneal edema, corneal opacification, and, consequently, impaired vision. At present, allogeneic corneal transplantation is the most effective way to treat corneal endothelial dysfunction. However, this procedure is limited by a global scarcity of healthy donors (Shimazaki et al., 2004).

A number of technologies have been developed to use cultured CECs as an alternative to donor corneal endothelium (Engelmann et al., 1988; Joyce and Zhu, 2004; Mimura et al., 2013; Proulx and Brunette, 2012; Sumide et al., 2006). However, it is extremely difficult to culture human CECs for long periods (Peh et al., 2011). This difficulty is encountered because cultured CECs easily lose typical CEC characteristics, by switching their phenotype from endothelial to fibroblastic (Okumura et al., 2013) in a process referred to as endothelial-to-mesenchymal transition (Roy et al., 2015), which limits the use of cultured CECs for the treatment of corneal endothelial disorders. To solve this problem, recent tissue engineering studies have focused on the development of alternative CECs from other cell types, such as the iris (Kikuchi et al., 2011) and corneal stroma (Hatou et al., 2013). More recent studies successfully induced human embryonic stem cells to develop into CEC-like cells (Song et al., 2016; Zhang et al., 2014). Given the recent rapid progress in the field of stem cell research, a method to produce CECs from induced pluripotent stem cells is likely to be developed in the near future.

Since pluripotent stem cells can differentiate into various cell types, CEC-specific markers are necessary for the evaluation of the final

Abbreviations: CEC, corneal endothelial cell; CE tissue, corneal endothelial tissue; HCEPs, human corneal endothelial progenitors; dHCEPs, differentiated human corneal endothelial progenitors; CAGE, cap analysis of gene expression; FANTOM, Functional Annotation of Mammalian Genome; tpm, tags per million.

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products. Moreover, to reproduce the developmental process of human CECs *in vitro*, it is also essential to understand the molecular dynamics of human CEC differentiation. Several studies demonstrated that the neural crest-derived periocular mesenchyme gives rise to corneal endothelium and stroma, trabecular meshwork, iris, ciliary body, and sclera (Cvekl and Tamm, 2004; Gage et al., 2005; Williams and Bohnsack, 2015). These studies revealed that PITX2 is required for the differentiation of the neural crest-derived periocular mesenchyme during early ocular development in mice (Gage et al., 2005). Mutations in *PITX2* are associated with Axenfeld-Rieger syndrome, which is characterized by dysgenesis of anterior segment, including corneal endothelium (Kozlowski and Walter, 2000; Lines et al., 2002). These observations indicate that PITX2 plays a crucial role in the development of the human neural crest-derived periocular mesenchyme. However, key regulators of human CEC lineage commitment from periocular mesenchyme remain to be elucidated. We previously isolated human corneal endothelial progenitors (HCEPs) from CECs, and successfully converted these HCEPs into differentiated HCEPs (dHCEPs) that had pump function similar to that of CECs (Hara et al., 2014).

Pursuing a comprehensive molecular understanding of human CECs and their differentiation process, here we explored transcriptome characteristics of human CECs, including HCEPs and dHCEPs, using cap analysis of gene expression (CAGE), which enabled us to monitor promoter activities at the genome-wide level (Shiraki et al., 2003). First, we identified specific markers of CECs by referring to the Functional Annotation of Mammalian Genome 5 (FANTOM5) expression atlas, which catalogs promoter activities in a wide variety of human tissue and cell samples (Forrest et al., 2014). Next, we identified transcription factors that are specifically expressed in CECs, which might control the cell fate and lineage commitment of CECs. Finally, we analyzed transcriptional dynamics during human CEC differentiation, and found that the majority of CEC-specific promoters are upregulated during differentiation. These findings may facilitate selective differentiation of CECs *in vitro*, and thereby accelerate the development of corneal regenerative medicine.

2. Materials and Methods

2.1. Preparation of Human Corneal Endothelial Samples for CAGE Analysis

The use of all human samples in this study adhered to the tenets of the Declaration of Helsinki. Research-grade corneoscleral rims and whole eye globes from cadaver human donors were obtained from SightLife (Seattle, WA, USA). Informed consent for eye donation to research was obtained from the next of kin of all deceased donors by SightLife.

2.1.1. Preparation of Human Corneal Endothelial Tissues

To obtain the freshest possible corneal endothelial samples, we recovered 36 corneal endothelial (CE) tissues within a few days following death (22 ± 13 h), and before shipping (Fig. S1a). Descemet's membranes with the corneal endothelial monolayer were carefully dissected from corneoscleral rims, using sterile surgical forceps, as described previously (Yoshihara et al., 2015). The stripped Descemet's membranes with endothelium were immediately transferred into RNAlater RNA Stabilization Reagent (QIAGEN Inc., Valencia, CA, USA). Among these tissue samples, three from healthy donors with high RNA quality were analyzed by CAGE.

2.1.2. Preparation of Cultured CECs, HCEPs, and dHCEPs

To cultivate human CECs, Descemet's membranes with their endothelium were treated with enzyme-containing cell detachment medium (Accutase; Life Technologies, Grand Island, NY, USA) at 37 °C for 30 min, and seeded onto culture dishes coated with 0.1 μg/cm^2 laminin-511E8 (Wako Pure Chemical Industries, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies), supplemented with 10% fetal bovine serum (FBS; Japan Bio Serum, Hiroshima, Japan) and 2 ng/mL basic fibroblast growth factor (bFGF; Wako Pure Chemical Industrials). CECs at the proliferation stage were collected and subcultured when they reached 70% confluence, and collected again when they reached 100% confluence.

HCEPs and dHCEPs were obtained according to previously described procedures (Hara et al., 2014). Briefly, the Descemet's membranes were stripped from the corneas in DMEM, and treated with Accutase at 37 °C for 30 min. The detached CECs were seeded at a density of 100–300 cells/cm^2 onto culture plates coated with 0.1 μg/cm^2 laminin-511E8. The medium was composed of DMEM/Nutrient Mixture F-12 (DMEM/F12; Life Technologies) containing 20% Knockout Serum Replacement (KSR; Life Technologies), 2 mM L-glutamine (Life Technologies), 1% non-essential amino acids (Life Technologies), 100 μM 2-mercaptoethanol (Life Technologies), 50 U/mL penicillin G, 50 μg/mL streptomycin (Life Technologies), and 4 ng/mL bFGF. The culture medium was changed every 2–3 days. When the cells reached 70% confluence, they were harvested with Accutase and passaged at ratios of 1:2–1:5. HCEPs were differentiated into mature CECs (*i.e.*, differentiated HCEPs: dHCEPs) on dishes coated with FNC coating mix (AthenaES, Baltimore, MD, USA). The differentiation medium consisted of DMEM supplemented with 10% FBS, 50 U/mL penicillin G, and 50 μg/mL streptomycin. The cells were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2 for 28 days.

2.1.3. RNA Preparation From CEC Samples

Total RNA was extracted from tissues or cells, using an miRNeasy Mini Kit (QIAGEN Inc.), according to the manufacturer's instructions. The quantity and quality of the extracted RNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) of each sample is shown in Table S1.

2.2. CAGE Analysis and Data Processing

2.2.1. CAGE Library Preparation

CAGE libraries were prepared from total RNA, as previously described (Kanamori-Katayama et al., 2011), using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) for reverse transcription, $NaIO_4$ for diol oxidation, biotin hydrazide (Vector Laboratories, Burlingame, CA, USA) for biotinylation, RNase I (Promega, Madison, WI, USA) for single-strand RNA digestion, streptavidin-coated magnetic beads (Dynabeads M-270 Streptavidin; Life Technologies) for biotinylated RNA/cDNA recovery, and an Agencourt AMPure XP Kit (Beckman Coulter, Brea, CA, USA) for purification and buffer exchange. After polyA tailing reaction using terminal transferase and dATP, cDNAs were blocked with ddATP. The resulting CAGE libraries were loaded on two lanes of a HeliScope single molecule sequencer (Helicos Biosciences, Cambridge, MA, USA). An overview of the sequencing data is presented in Table S1. All CAGE sequence data analyzed in this study were deposited to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/index_e.html) under accession number DRA005836.

2.2.2. Annotation of Promoters and Differential Expression Analysis

After base calling, raw reads containing base-order addition artifacts, and other low-quality reads, were removed using an SMS filter program supplied by Helicos. In addition, reads shorter than 20 nucleotides and longer than 70 nucleotides were removed. These filtered reads were mapped to the human genome sequence (hg19), using Delve (Djebali et al., 2012) and the MOIRAI pipeline platform (Hasegawa et al., 2014). Mapped reads (tags) were counted with respect to the robust peaks identified in the FANTOM5, which was used as a reference for promoter regions (Forrest et al., 2014). On the basis of the total number of tags, CAGE peaks associated with a single gene were labeled as p1, p2, and so forth. For example, p1@PITX2 corresponds to one of the alternative promoters of *PITX2,* which has the highest tag counts in the FANTOM5. In this study, we regarded p1–p3 as major promoters. Raw tag counts

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