Generation and validation of a PITX2–EGFP reporter line of human induced pluripotent stem cells enables isolation of periocular mesenchymal cells

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PITX2 (Paired-like homeodomain transcription factor 2) plays important roles in asymmetric development of the internal organs and symmetric development of eye tissues. During eye development, cranial neural crest cells migrate from the neural tube and form the periocular mesenchyme (POM). POM cells differentiate into several ocular cell types, such as corneal endothelial cells, keratocytes, and some ocular mesenchymal cells. In this study, we used transcription activator–like effector nuclease technology to establish a human induced pluripotent stem cell (hiPSC) line expressing a fluorescent reporter gene from the PITX2 promoter. Using homologous recombination, we heterozygously inserted a PITX2–IRES2–EGFP sequence downstream of the stop codon in exon 8 of PITX2. Cellular pluripotency was monitored with alkaline phosphatase and immunofluorescence staining. EGFP and PITX2 expression, as indicated by immunoblotting and simultaneous zones. Using a combination of previously reported methods, we induced PITX2 in the hiPSC line and observed simultaneous EGFP and PITX2 expression, as indicated by immunoblotting and immunofluorescence staining. PITX2 mRNA levels were increased in EGFP-positive cells, which were collected by cell sorting, and marker gene expression analysis of EGFP-positive cells induced in self-formed ectodermal autonomous multizones revealed that they were genuine POM cells. Moreover, after 2 days of culture, EGFP-positive cells expressed the PITX2 protein, which co-localized with forkhead box C1 (FOXC1) protein in the nucleus. We anticipate that the PITX2–EGFP hiPSC reporter cell line established and validated here can be utilized to isolate POM cells and to analyze PITX2 expression during POM cell induction.

Neural crest cells (NCCs)² are multipotent stem cells generated at the border between the neural tube and surface ectoderm during early embryonic development in vertebrates (1). In eye development, cranial NCCs migrate to form the periocular mesenchyme (POM). POM cells in turn differentiate into a wide variety of cells, such as corneal endothelial cells (2), keratocytes, iris stromal cells, ciliary muscle cells, trabecular meshwork cells, and scleral cells (3, 4). In addition, peripheral tissues of the eyes, such as cartilage, bone, dermis, and fat, which are connected to the extraocular muscles, also originate from POM cells (5).

PITX2 (paired-like homeodomain transcription factor 2) is one of the homeobox transcription factors that play key roles during embryogenesis. PITX2 is crucial in left–right asymmetry in visceral organs (6), such as the heart (7–9), lungs (7), gut (8, 10), and stomach, as well as in eye development (11). In the eyes, PITX2 is expressed in the cranial NC-derived POM cells, together with other transcription factors, such as FOXC1, FOXC2 (12), and LMX1B (13), and plays important roles in ocular anterior segment development (14). Specifically, PITX2 is involved in the development of corneal endothelial cells (15), keratocytes (16), iris stromal cells (17), ciliary muscles, trabecular meshwork cells, scleral cells, mesenchymal cells of the ocular glands, and peripheral connective tissues connected to the extraocular muscles (18). Mutation of PITX2 or FOXC1 causes Axenfeld–Rieger syndrome, which manifests with dysgenesis of the anterior segment of the eyes as well as mild tooth malformation and craniofacial dysmorphism (19, 20).

It has been suggested that POM cells can be induced from human induced pluripotent stem cells (hiPSCs) (21). We recently reported that hiPSCs form self-formed ectodermal autonomous multizones (SEAMs) from which ocular cells, such as corneal epithelial cells, conjunctival epithelial cells, lens cells, retinal cells, and NCCs, can be derived (22, 23).

² The abbreviations used are: NCC, neural crest cell; POM, periocular mesenchyme; hiPSC, human induced pluripotent stem cell; SEAM, self-formed ectodermal autonomous multizone; TALEN, transcription activator–like effector nuclease; Eν, embryonic day ν; MEF, mouse embryonic fibroblast; ALP, alkaline phosphatase; qRT-PCR, quantitative RT-PCR; DM, differentiation medium; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PE, phycoerythrin.
various types of cells mimic their differentiation process to form a whole eye structure in vitro. We expect that SEAMs contain PITX2-expressing POM cells differentiated from NCCs. However, it is nearly impossible to isolate PITX2-expressing POM cells from the various cell types in culture systems without a reporter line, because POM cell-specific surface markers have not been reported to date.

Transcription activator–like effector nucleases (TALENs) are restriction enzymes that generate site-specific double-strand breaks in DNA, with lower nonspecific cleavage activity (24) than CRISPR-Cas (25), through binding of a TAL effector to specific DNA regions (26). The double-strand breaks are repaired through nonhomologous end joining or homologous recombination. TALENs can be used for specific gene knockout or knockin. We previously demonstrated that a p63 knockin reporter line generated with TALEN technology could be used for detailed analysis and isolation of p63-positive cells in SEAMs (27).

Here, we report the generation of a PITX2 reporter line of hiPSCs harboring an IRES2–EGFP sequence, using TALEN technology. We validated the reporter line in a system in which PITX2 expression is induced in pluripotent stem cells. In addition, we were able to isolate and analyze POM cells. The PITX2 reporter hiPSC line generated in this study allows robust induction and isolation of POM-derived cells and insights into the detailed mechanisms of induction of POM cells and POM cell–derived cells.

Results

PITX2 is expressed in POM cells in the mouse embryo

We evaluated Pitx2 expression in POM cells in mouse embryos at E10.5 (Fig. S1A) and E12.5 (Fig. S1B). At both stages, cells ranging from the pericocular sites to primordial cells of the cornea were positive for Pitx2 and Foxc1, but negative for Sox10, a negative marker of POM cells. This finding indicated that POM cells exist in the pericocular sites in E10.5 and E12.5 mouse embryos.

Evaluation of GFP fluorescence intensity driven by the PITX2 promoter

To achieve strong GFP fluorescence upon forced expression in 293T cells, various GFP variants, polycistronic sequences, and polyadenylation (poly(A)) signals were evaluated. First, three GFPs—EGFP, EmGFP, and TurboGFP—were inserted downstream of the PITX2 and 2A peptide sequences in pEF5/FRT/V5-DEST. There were no obvious differences in intensity between these three GFPs (Fig. S2A). As polycistronic sequences, 2A peptides and IRES2 were evaluated. Fluorescence intensity was stronger when EGFP was located downstream of IRES2 than when it was downstream of the 2A peptide sequences (Fig. S2B). As for polyadenylation, poly(A) signals of bovine growth hormone, herpes simplex virus–thymidine kinase, SV40, PITX2 3′-UTR, and β-actin 3′-UTR were evaluated. SV40 and PITX2 3′-UTR yielded slightly stronger EGFP intensity than the other poly(A) signals (Fig. S2C).

Based on our findings, we used a donor vector containing EGFP, IRES2, and SV40 as the GFP variant, polycistronic sequence, and polyadenylation signal, respectively, for establishing a PITX2–GFP reporter hiPSC line.

Design of a TAL effector and donor vector

There are six splice variants of PITX2 and three isoforms (Fig. 1A). Exon 8 is common to all PITX2 variants. Thus, we added the artificial sequences downstream of PITX2 exon 8. As shown in Fig. 1B, a TAL effector recognition site in the left arm was located immediately upstream of a stop codon of PITX2, and a right-arm recognition site was designed after the PITX2 stop codon. The donor vector was designed so that IRES2–EGFP–SV40 poly(A) followed the left arm of PITX2 with a silent mutation to avoid the generation of double-strand breaks after successful site-specific double-strand break generation by the TALEN.

Generation of a PITX2–EGFP knockin reporter hiPSC line

After electroporation of the TALEN vector and donor vector into 201B7 hiPSCs, the cells were seeded on DR4 mouse embryonic fibroblasts (MEFs) for drug selection. Knockin cells were screened on G418 sulfate as outlined in Fig. 2A. Based on PCR results, colony 8 (IRES2) was selected for further re-cloning analysis (Fig. 2B). Colonies 5 and 7 (2A) seemed to be successfully transfected; however, they were not further analyzed because the 2A peptides yielded lower EGFP intensity as shown in Fig. S2B. Colony 8 was re-cloned, and six colonies were analyzed by PCR, which revealed that the construct was heterozygously introduced in all six colonies. Colony 8–2 produced a slightly stronger band intensity than the other colonies (Fig. 2C) and was therefore chosen as the best candidate PITX2–EGFP hiPSC reporter line for further analysis. The genome sequence of this line was confirmed using Sanger sequencing (Fig. S3).

Pluripotent stem cell markers and typical SEAM phenotypes in the PITX2–EGFP knockin reporter line

After passaging the PITX2 knockin hiPSC line for feeder-free culture, the cells formed round, normal colonies on iMatrix-511, as shown in Fig. 3A. In an alkaline phosphatase (ALP)–staining assay, PITX2 knockin hiPSC clone 8–2 showed a staining intensity and color similar to those of 201B7 WT hiPSCs (Fig. 3B). Expression of the pluripotent markers NANOG, OCT3/4, TRA-1–60, and SSEA-4 was evaluated by immunofluorescence analysis using specific antibodies. All these markers showed strong expression from the center to the borders of the colonies (Fig. 3C). We successfully induced SEAM structures consisting of four zones using the PITX2 knockin hiPSC line as reported previously (22, 23). After SEAM induction, markers of corneal epithelial cells (p63, PAX6) lens cells (p63, α-crystallin), neuroretina (CHX10), and retinal pigment epithelial cells (MITF) were stained (Fig. 3D). The cells in zone 3 were p63- and PAX6-positive and showed cobblestone morphology, which indicated that they were corneal epithelial cells. The aggregated cells at the end of zone 2 were p63- and α-crystallin-positive lens cells. The cells in the inner area of zone 2 were CHX10-positive neuroretinal cells. The cells aggregated in the outer area of zone 2 were MITF-positive retinal pigment epithelial cells. All these structures were similar to those induced in 201B7 hiPSCs. We also evaluated gene expression patterns in SEAMs (Fig. 3E). Expression of TUBB3, a neuron marker, was substantially higher in zone 1. Expression of the neural crest cell marker SOX10 and the neural retina marker RAX was higher in zone 2. PAX6 was expressed in all
zones. Epithelial markers DN-p63, CDH1, and KRT18 were highly expressed in zones 3 and 4. The lens cell marker CRYAA was the most strongly expressed in zones 3 and 4. This expression pattern was similar to that of 201B7 hiPSCs (22).

Validation of the PITX2–EGFP knockin reporter line

To confirm that EGFP is expressed in the PITX2 knockin hiPSC line, POM cells were induced by a combination of reported induction methods (28–31) (Fig. 4A). Aggregated cells were detected ~20 days after the start of induction (Fig. 4B). The aggregated cells showed EGFP signals at day 20 (Fig. 4C). We determined EGFP and PITX2 protein expression levels using whole cell lysates by Western blotting analyses. PITX2 expression in knockin iPSCs was nearly the same as that in clone 8-2 PITX2 knockin iPSCs (Fig. 4D), and we observed robust protein expression of EGFP in PITX2 knockin iPSC clone 8-2. Next, we conducted immunofluorescence staining using anti-PITX2 antibody to evaluate whether EGFP and PITX2 are expressed simultaneously. As shown in Fig. 4E, EGFP fluorescence and PITX2 fluorescent staining were detected simultaneously. Next, we sorted and collected EGFP-positive and -negative cells by FACS (Fig. 4F). We analyzed PITX2 expression levels by quantitative RT-PCR (qRT-PCR). The population of EGFP-positive cells exhibited high PITX2 expression, whereas EGFP-negative cells hardly expressed PITX2. Moreover, FOXC1 and TFAP2B, which are markers of POM cells, were significantly more strongly expressed in EGFP-positive cells when compared with EGFP-negative cells. Conversely, SOX10, which is a negative marker of POM cells, was more strongly expressed in EGFP-negative than in EGFP-positive cells. On the other hand, the POM markers FOXC2, LMX1B, NGFR, LMX1B, and COL8A2 were not highly expressed in EGFP-positive cells (Fig. 4G).

Isolation and characterization of POM cells

To acquire more genuine POM cells, we tried a SEAM induction method (Fig. 5A). Typical, aggregated cells emerged in SEAM zone 2 after 14 days of induction (Fig. 5B). EGFP signals were confirmed at the location of aggregated cells in SEAM zone 2 (Fig. 5C). After sorting EGFP-positive cells (Fig. 5D), they were analyzed for marker expression by qRT-PCR. All positive POM cell markers were significantly increased in EGFP-positive compared with EGFP-negative cells, and there was no difference of SOX10 expression level between them (Fig. 5E), which revealed that the EGFP-positive cells were POM cells. They were cultivated for 2 days in a culture plate using differentiation medium (DM) with Y-27632, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and retinoic acid (Fig. 5F). Cells expressed PITX2 protein, which co-localized with FOXC1 protein in the nucleus (Fig. 5G).
Discussion

Various GFP variants, polycistronic sequences, and poly(A) signals were evaluated in a stepwise manner to establish a PITX2–EGFP knockin hiPSC reporter line with optimum GFP expression. The GFP variants EGFP, EmGFP, and TurboGFP did not show a difference in fluorescence intensity. Unexpectedly, the IRES2 sequence yielded stronger EGFP fluorescence than the 2A peptides. The 2A peptides were added at the C terminus of PITX2, and the proline added at the N terminus of GFP might have affected GFP expression or fluorescence. Alternatively, unknown mechanisms might determine the compatibility between target gene and following polycistronic sequences. The various polyadenylation signals tested yielded slightly different EGFP expression. Interestingly, among them, poly(A) of SV40 and PITX2 3’-UTR had the strongest ability to stabilize PITX2 mRNA.

The efficiency of PITX2–IRES2–EGFP knockin was 1 of 12. This was more or less as expected, but there is room for improving the knockin efficiency, for example by increasing the vector concentrations and optimizing the electroporation program.

The reporter line established in this study showed normal pluripotency based on ALP staining and immunofluorescence staining of the markers analyzed in this study. We confirmed that a PITX2 knockin hiPSC line formed typical SEAMs and
showed a robust ability to induce lens cells, neuroretinal cells, and retinal pigment cells, although they induced less corneal epithelial cells than 201B7 hiPSCs did (Fig. 3D). Such a difference in differentiation tendency often occurs among pluripotent stem cell lines (32).

We were able to establish PITX2-expressing cells by combining published approaches: NC induction by WNT and low BMP signaling (28), corneal endothelial cell induction by EGF treatment, and FGF signaling for POM cell-derived tissue induction (29). Unfortunately, the cells did not show perfect gene expression patterns of POM cells, although they showed high FOXC1 mRNA expression and low SOX10 mRNA expression, which are characteristic for POM cells (12, 33). However, PITX2-expressing cells induced by the SEAM method did show perfect gene expression patterns of POM cells based on qRT-PCR results (Fig. 5E). Moreover, COL8A2 and COL8A1, which are markers of POM and corneal endothelial cell, were significantly increased in EGFP-positive cells. This might indicate that they are POM cells and have a potential to differentiate into POM-derived cells. In the near future, we would like to utilize this cell line to further analyze POM cells in SEAM-inducing and other culture conditions.

In conclusion, we successfully generated and validated a PITX2–IRES2–EGFP knockin hiPSC line, and we were able to isolate PITX2-expressing POM cells. These cells showed higher POM marker expression than EGFP-negative cells. The POM cells sorted in this study are a reliable tool for detailed analysis of POM-derived ocular cells. Our reporter line provides a technical platform for testing induction methods and for detailed analysis of PITX2-expressing cells and their derivatives.

**Experimental procedures**

**Immunostaining of mouse embryos**

All animal experimental protocols in this study were in accordance with the Association for Research in Vision and...
Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research, with prior approval from the Animal Ethics Committee of Osaka University. E10.5 and E12.5 ICR mice were purchased from Japan SLC (Sizuoka, Japan). After the mice were euthanized with pentobarbital sodium, they were perfused with PBS. Mouse embryos were collected and were also euthanized. They were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan), frozen on dry ice, and stored at −80 °C. The mouse embryos were sectioned and incubated with blocking solution (5% normal donkey serum, 0.3% Triton X-100 in TBS). Then the sections were incubated with primary antibodies against PITX2 (Ab55599; Abcam, Cambridge, MA), FOXC1 (8758S; Cell Signaling Technology), and SOX10 (sc-17342; Santa Cruz Biotechnology) at 4 °C overnight, followed by secondary antibodies conjugated with Alexa Flour 488, Alexa Flour 568, Alexa Flour 594, and Alexa Flour 647 (Thermo Fisher Scientific) for 1 h. The sections were counterstained with Hoechst 33342 (Thermo Fisher Scientific) and visualized and imaged under an Axio Observer D1 microscope (Carl Zeiss).

**Analysis of fluorescence intensity**

293T cells were seeded in a 6-well plate at 3.0–4.0 × 10^5 cells/well. Constructs for forced expression in 293T cells were synthesized using Gateway LR Clonase II (Thermo Fisher Scientific) and the pEF5/FRT/V5-DEST vector (Thermo Fisher Scientific). FuGENE HD transfection reagent (Promega, Madison, WI) and 1.3 µg of each plasmid harboring PITX2, and EGFP, EmGFP, or TurboGFP as a fluorescent reporter, 2A or IRES2 sequences as a polycistronic sequence, and bovine...
growth hormone, herpes simplex virus-thymidine kinase, SV40, PITX2 3'-UTR, and β-actin 3'-UTR as a polyadenylation signal were used in the experiments. After 24 h, fluorescence was evaluated and imaged using the Axio Observer D1. The experiments regarding recombinant DNA and genome-editing were approved by the research ethics committee of Osaka University and were performed in accordance with guidelines of Osaka University.

hiPSC culture

The hiPSC line 201B7 was kindly provided by the Center for iPS Cell Research and Application, Kyoto University (Kyoto, Japan). The cells were cultured on dishes seeded with MEFs in Dulbecco’s modified Eagle’s medium/F-12 (Thermo Fisher Scientific) supplemented with 20% knockout serum replacement (Thermo Fisher Scientific), 0.1 mM nonessential amino acids (Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific), and 4 ng/ml bFGF (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan), with or without 10 μM Y-27632 (Fujifilm Wako Pure Chemical Corporation), which is selective inhibitor of Rho-associated coiled coil-forming protein kinases, to avoid apoptosis induction by electroporation. For feeder-free culture, the cells were cultured on dishes coated with iMatrix-511 (0.5 g/cm²; Nippi, Tokyo, Japan) in StemFit medium (Ajinomoto, Tokyo, Japan).

Construction of TALEN plasmids and the donor vector

PITX2 TALEN-L/R sequences were synthesized using GeneART Precision TALs (Thermo Fisher Scientific). Basic structure of the donor vector harboring an IRES2 (2A)-EGFP-SV40 poly(A)-neomycin cassette flanked by PITX2 homology arms was constructed by FASMAC (Kanagawa, Japan).
Establishment of the PITX2–IRES2–EGFP knockin hiPSC line

TALEN plasmids (2.5 μg) and donor vector (5 μg) were mixed in the solution included in the P3 primary cell 4D-Nucleofector™ X kit (Lonza, Basel, Switzerland) and were electroporated into 1.5 × 10^6 hiPSCs using program CB150 in the 4D-Nucleofector™ system (Lonza). The electroporated cells were seeded on DR4 MEFs (ASF-1001; Applied StemCell, Milpitas, CA) and were cultured for 5 days for recovery. The cells were cultured in the presence of G418 sulfate for 8 days and then in the absence of G418 sulfate for 4 days. Then the cells were picked up and transferred to a 12-well plate and cultured for 11 days. The cells were used for the screening of knockin colonies by end point PCR. Then the candidate knockin clone 8 was reseeded and screened as described above. The candidate knockin clone 8-2 was transferred to a 10-cm dish. Eventually, we used the heterozygous knockin clone 8-2, whose knockin sequence was confirmed by sequencing on an Applied Biosystems 3100 Genetic Analyzer (Thermo Fisher Scientific).

End point PCR for the screening of knockin colonies

Candidate clones were picked up and cultured in single wells of a 96-well plate. After 1 day, floating cells were harvested and lysed. The genomic DNA was extracted with a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany) and was used for end point PCR using Tks Gflex™ DNA polymerase (Takara Bio, Shiga, Japan) and two primer pairs. The primers were as follows: primer pair 1: forward: TGCATTTCTAGTTGTTGTTTGTC; reverse: AGTGTCTCCTGTGGATGCAATGA, thermal cycles: 94 °C for 1 min and 30 cycles of 98 °C for 10 s, 60 °C for 15 s, 68 °C for 30 s; primer pair 2: forward: TAGTAATCTGGACTGGCCATCT, reverse: AGTTTTCTCTGGTGGAATGCAATGA, thermal cycles: 94 °C for 1 min and 30 cycles of 98 °C for 10 s, 60 °C for 15 s, 68 °C for 2 min.

Sanger sequencing

The cells were harvested and genomic DNA was extracted using a NucleoSpin® tissue kit (Macherey-Nagel). Sanger sequencing was conducted using Applied Biosystems 3730 DNA Analyzer and a BigDye™ Terminator version 3.1 cycle sequencing kit (both from Thermo Fisher Scientific), according to the manufacturer’s instruction.

ALP staining

ALP staining was conducted using a TRACP and ALP double-stain kit (Takara Bio). Briefly, the cells were washed with PBS, fixed with fixation solution, and washed with water. The cells were treated with ALP substrate solution at 37 °C for 15 min and then washed twice with water. The cells were photographed using an EVOS FL auto imaging system (Thermo Fisher Scientific).

Immunofluorescence staining

hiPSC colonies, SEAMs, and POM cells were fixed with 4% paraformaldehyde and permeabilized with solution including 1% normal donkey serum, 0.3% Triton X-100 in TBS at 4 °C for 3 days or overnight. The colonies were treated with blocking solution (5% normal donkey serum, 0.3% Triton X-100 in TBS) for 1 h and then with primary antibodies against NANOG (Ab62734; Abcam), OCT3/4 (Ab19857; Abcam), SSEA-4 (MC-81370; BioLegend, San Diego, CA), and TRA-1–60 (330614; Santa Cruz Biotechnology), α-crystallin (SPA-224; Stressgen Biotechnologies, San Diego, CA), CHX10 (sc-21690; Santa Cruz Biotechnology), and MITF (X1405M; Exalpha Biologicals, Shirley, MA) overnight. The POM cells were treated with primary antibody against PITX2 (Ab55599; Abcam) and FOXC1 (8758S; Cell Signaling Technology). Subsequently, the colonies and POM cells were labeled with secondary antibodies conjugated to Alexa Fluor 568, 594, 647, Plus 594, or Plus 647 (Thermo Fisher Scientific) for 1 h, counterstained with Hoechst 33342 (Thermo Fisher Scientific), and fluorescence images were acquired using an Axio Observer D1 or FLUOVIEW FV3000 (Olympus, Tokyo, Japan).

SEAM induction in hiPSCs

SEAMs were induced as described in our previous reports (22, 23). Briefly, hiPSCs were seeded in a 6-well plate coated with iMatrix-511 at 1500–4500 cells/well and cultivated in StemFit medium, with a medium change every 2–3 days. After 10 days, the medium was replaced with DM (Glasgow’s minimum essential medium; Thermo Fisher Scientific) supplemented with 10% knockout serum replacement, 1 mM sodium pyruvate (Thermo Fisher Scientific), 0.1 mM nonessential amino acids (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 1% penicillin–streptomycin solution (Thermo Fisher Scientific), and 55 μM 2-mercaptoethanol (Thermo Fisher Scientific) or monothioglycerol (Fujifilm Wako Pure Chemical Corporation), and the cells were cultivated for 4 weeks, with a medium change every 2–3 days. Then the medium was replaced with corneal DM consisting of DM and Cnt-20 or Cnt-PR without EGF and FGFR2 (CELLnTEC Advanced Cell Systems, Bern, Switzerland) at 1:1, plus 20 ng/ml KGF (Fujifilm Wako Pure Chemical Corporation), 10 μM Y-27632, and 1% penicillin–streptomycin. For immunofluorescence staining of α-crystallin, CHX10, and MITF, the medium was directly replaced with corneal epithelium maintenance medium (Dubbecco’s modified Eagle’s medium/F-12 (2:1), Thermo Fisher Scientific) containing 2% B27 supplement (Thermo Fisher Scientific), 1% penicillin–streptomycin solution, 20 ng/ml KGF (Fujifilm Wako Pure Chemical Corporation), and 10 μM Y-27632 instead of corneal DM. The cells were cultivated for 4 weeks with a medium change every 2–3 days.

Induction of PITX2

Cranial neural crest and neural crest cells were induced from hPSCs as previously described (28–31). Using these cells as a reference, we established a POM cell induction method. hiPSCs were seeded in a 6-well plate coated with iMatrix-511 (0.5 μg/cm²) at 1300–1500 cells/well. After 8 days of cultivation in StemFit medium, the medium was replaced with DM to start induction, and the cells were cultured for 2 days. Then the medium was replaced with DM containing 0–10 ng/ml BMP4 (R&D Systems, Minneapolis, MN) and 0–10 μM CHIR99021.
PITX2 reporter line from human pluripotent stem cells

(Sigma–Aldrich), and the cells were cultured for 2 days. Then the medium was replaced with DM without any additional reagents, and the cells were cultured for 4 days. Then the medium was replaced with DM containing 20 ng/ml EGF (R&D Systems) and 10 ng/ml bFGF, and the cells were cultured until analysis.

Western blotting analysis

The cells were lysed with radioimmune precipitation assay lysis and extraction buffer (Thermo Fisher Scientific) containing protease inhibitor mixture set I (Fujifilm Wako Pure Chemical Corporation) and sonicated. Protein concentrations were measured with a BCA protein assay kit (Thermo Fisher Scientific). The samples were analyzed using the WES system (ProteinSimple, San Jose, CA). Antibodies against GAPDH (sc-32233; Santa Cruz Biotechnology), PITX2 (Ab55599; Abcam), and GAPDH (sc-8334; Santa Cruz Biotechnology) were used. Other reagents, including secondary antibodies, were used following the manufacturer’s instructions.

Flow cytometry

hiPSCs were cultured for 20 days and then dissociated with Accutase for 30 min. Accutase was removed by centrifugation, and the cells were resuspended in PBS. The cells were analyzed and sorted using an SH800 cell sorter (Sony, Tokyo, Japan). Aggregated cells were gated and excluded. Single cells were expanded in R-phycoerythrin (PE) and FITC to exclude autofluorescent cells. The cells were selected using polygon gates; the cells in the purple polygon were counted as EGFP-positive cells, whereas cells in the blue polygon were considered EGFP-negative cells.

qRT-PCR

Total RNA was extracted from cells that were manually harvested from each SEAM zones and from EGFP-negative cells. The cells were manually harvested from each SEAM zones and from EGFP-negative cells. The cells were manually harvested from each SEAM zones and from EGFP-negative cells. Gene expression was normalized using GAPDH as an internal control.

Cultivation of EGFP-positive cells

EGFP-positive cells were sorted by FACS. The cells were attached to a plate coated with 0.5 μg/cm² iMatrix-511 and 1.56 μg/cm² fibronectin by centrifugation at 210 × g for 4 min. The cells were cultivated in DM with 10 μM Y-27632, 20 ng/ml EGF, 10 ng/ml bFGF, and 50–100 nm retinoic acid. After 2 days of cultivation, phase contrast images of the cells were captured using the Axio Observer D1.

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