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Lens epithelial cells-induced pluripotent stem cells as a model to study epithelial-mesenchymal transition during posterior capsular opacification

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ABSTRACT

The overall goal was to generate an epithelial-mesenchymal transition (EMT) model using lens epithelial cells-induced pluripotent stem cells to elucidate EMT-regulatory factors during posterior capsular opacification (PCO). For this purpose, the mouse lens epithelial cells-derived mesenchymal cells were reprogrammed to induced pluripotent stem cells (iPSC) and differentiated to lens epithelial cells to be used to determine regulatory factors during EMT. Lens epithelial cells from one-month-old C57BL/6 mice were transitioned to mesenchymal cells in culture, and were reprogrammed to iPSC by delivering reprogramming factors in a single polycistronic lentiviral vector (co-expressing four transcription factors, Oct 4, Sox2, Klf4, and Myc). iPSC were differentiated to epithelial cells by a three-step process using noggin, basic fibroblast growth factor (bFGF), bone morphogenetic protein 4 (BMP4) and Wnt-3. At various time points, the cells/clones were immunocytochemically analyzed for epithelial cell markers (Connexin-43 and E-cadherin), mesenchymal cell markers (Alpha-smooth muscle actin), stem cell markers (Sox1, Oct4, SSEA4 and Tra60) and lens-specific epithelial cell markers (αA- and βA3/A1-crystallins).

By increasing the number of genetic transductions, the time needed for generating iPSC from lens mesenchymal cells was reduced, successfully reprogrammed epithelial/mesenchymal cells into iPSC, and re-transformed iPSC into lens epithelial cells by the growth factors' treatment. The epithelial cells could serve as a model system to elucidate regulatory factors involved during EMT to therapeutically stop it.

1. Introduction

Among the lens epithelial and fiber cells, the anterior monolayer epithelial cells proliferate into fiber cells at the equator, and then fiber cells move to the center and withdraw from the cell cycle, elongate and terminally differentiate into secondary fiber cells [1]. Factors present in aqueous humor and vitreous regulate the epithelial cell proliferation and fiber cell differentiation, respectively [2,3]. Fibroblast growth factors (FGF) induce both epithelial cell proliferation and fiber cell differentiation by MAPK/ERK1/2-signaling pathway [4].

A common complication of cataract surgery is the Posterior Capsular Opacification (PCO, also known as a secondary cataract) with 20–40% incidence rate [5]. During the PCO, the vision is affected because the remaining epithelial cells after surgery undergo wound healing response with proliferation and differentiation to produce α-smooth muscle actin (α-SMA)-expressing myofibroblasts, collagen deposit, and fiber cell degeneration. The epithelial and mesenchymal cells differ phenotypically, and even in their functions. Epithelial cells are connected to each other by tight junctions and adherent junctions, contain apico-basal polarity, exhibit polarization of actin cytoskeleton, and are bound to the basal lamina at their basal surface. In contrast, mesenchymal cells have irregular shaped morphology, and interaction through the focal points [6]. Further, the epithelial cells express high levels of E-cadherin (responsible for cell-cell adhesion), whereas in contrast, its down-regulation and high levels of expression of N-cadherin, fibronectin, vimentin and α-smooth muscle actin [α-SMA] occurs in mesenchymal cells [7]. Reorganization of α-SMA, vimentin, fibronectin, and actin cytoskeleton are needed for the motility of mesenchymal cells [8].

EMT plays a central role in the pathogenesis of PCO, and also during fibrotic disorders in the kidney, lung, liver, eye, serosal membranes and in cancer metastasis [9]. The down-regulation of E-cadherin affects *trans*-epithelial junctions and also the fibrotic process [10], and fibronectin (a major mesenchymal cell marker) is involved in the conversion of various cell types to myofibroblasts under pathological conditions [11].

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Transforming growth factorβ (TGFβ) induce EMT in a variety of cells [12], and the level of TGFβ2 (a latent isoform of TGFβ and an inducer of EMT in the aqueous humor) is elevated following cataract surgery [13]. TGFβ2, on binding to cell surface receptor, induces the major Smad-mediated canonical pathway, and also the minor non-canonical pathway mediated by ERK, MAPK, and Notch pathways [14].

The blocking of the EMT is key to preventing the PCO development. The present YAG laser treatment of PCO shows severe complications such a retinal detachment and macular edema [15]. A gene therapy approach is proposed to eradicate post-cataract surgery-proliferating epithelial cells by introducing a cytotoxic gene in the capsular bag while respecting the surrounding ocular tissues [16]. Another potential approach for the PCO prevention would be to therapeutically block the EMT based on its regulatory factors.

The reprogramming of somatic cells to iPSC using classic Yamanaka factors holds a great promise for bringing regenerative medicine into a clinical setting [17]. This approach has been used in mice [18,19] and in humans [20]. Similarly, the lens progenitor cells and lentoid bodies were generated from either human embryonic stem cells [21] or from human iPSC [22], and cataractous lenses [23] to gain knowledge about lens development, mechanism of cataractogenesis, and drug screening for different types of cataracts. Recently, following the removal of the cloudy lens in animals and in a small trial with human infants, the lens grew from their own stem cells [24]. These reports enhanced interest to utilize lens progenitor cells to gain information about the mechanism of EMT transition and its regulatory factors. We propose that the iPSC-derived epithelial cells and their *in vitro* differentiation to mesenchymal cells could be used as a model system to elucidate EMT regulatory mechanism, and allow screening of a variety of drugs to treat PCO.

2. Materials and methods

2.1. Productions of iPSC from mouse lens epithelial cells

Lenses from one-month-old C57BL/6 mice were cultured in medium199 with 20% fetal bovine serum (FBS) and 1% antibiotics (Gibco® Antibiotic-Antimycotic; containing penicillin, streptomycin, and Amphotericin B) for 48 h or longer until epithelial cells started to grow away from lens tissues. Next, the epithelial cells were transferred to another 6-well plate and cultured in the above medium containing reduced (10%) fetal bovine serum for five days until mesenchymal cell transition (Cells changed from classic spindle shape to an irregular form). These cells were reprogrammed by delivering twice reprogramming factors at 24 h intervals in a single virus using 2A “self-cleaving” peptides, using a single polycistronic lentiviral vector co-expressing four transcription factors (Oct 4, Sox2, Klf4, and Myc) to yield iPSC [25]. The cells were grown for an additional 8–10 days and at various time points, either the cells or clones were immunocytochemically analyzed. Next, the iPSC were differentiated to lens epithelial cells as described below.

2.2. Viral preparation and infection

To generate FUW-SOKM-harboring lentiviruses (LvSOKM) [25], 293T cells (3×10^6 cells per 10 cm dish) were co-transfected with a mix of 10 μg of pMD2.G plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelop, 10 μg of psPAX2 packaging plasmid and 12 μg of the FUW-SOKM [(Addgene plasmid 20325), Cambridge, MA], a lentiviral Sox2-P2A-OCT4-T2A-Klf4-E2A-cMyc-harboring shuttle, using lipofectamine 2000 (Invitrogen, Grand Island, NY), and following the manufacturer's instructions. The medium was changed to DMEM (Invitrogen, Grand Island, NY) after 24 h. The viral preparation and infection was done similar to as described earlier [26].

2.3. Immunofluorescence imaging

Cells in culture at the time points of day 0 and day 5 for the epithelial-mesenchymal transition, and the iPSC clones were fixed in 4% paraformaldehyde at room temperature, and immunocytochemically examined for epithelial markers (Connexin-43 (dilution1:100) and E-cadherin (1:100), mesenchymal markers (*Alpha*-smooth muscle actin {dilution1:200}, N-cadherin), and lens specific markers (CRYAB) and stem cell markers (Sox1, Oct4, SSEA4). The following individual primary antibodies were used: Sox1 (rabbit mAB, dilution 1:400), SSEA4 (mouse mAB, 1:500) and Oct4 (rabbit mAB, dilution 1:400), (Cell Signaling, Danvers, MA). Next, the cells were washed 3X in PBS, and incubated with a secondary antibody for 1 h in the dark. The following secondary antibodies were used: goat anti-rabbit IgG, AlexaFluor 488-conjugate (dilution 1:1000) and goat anti-mouse IgG, AlexaFluor 594-conjugate (dilution 1:1000) (ThermoFischer, Waltham, MA). The cells were washed 3X in PBS, incubated with Hoechst nuclear stain for 10 min, washed again in PBS, and mounted on to glass slides with a mounting medium (Fluromount-G, Southern Biotech, and Birmingham, AL). Mouse IgG was used as a negative control at the same protein concentration as that of the primary antibodies. One-way ANOVA was used for quantification of the average fluorescence intensity distribution is indicated for each protein in the graphs using graphpad prism.

2.4. Quantitative real-time PCR (qRT-PCR)

RNA was isolated from cells/clones by TRizol method (Invitrogen, Carlsbad, USA). The mRNA was reverse-transcribed using cDNA synthesis kit, and the mRNA levels were measured by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer's protocol. The list of the primers used are described below:

Vimentin: Forward: TTCTCTGGCACGTCTTGACC.
Reverse: CTCCTGGAGGTTCTTGGCAG.
Connexin 43: Forward: TGATTTCCCTGACGACAGCC.
Reverse: GTTGAGTACCACCTCCACGG.
E-Cadherin Forward: TGACTCTTGAAGGCTGTCGTC.
Reverse: ACGGTCGTTCGAATCCTAGC.
α-Smooth muscle.
Actin: Forward: GTCCACCGCAAATGCTTCTA.
Reverse: TTCCTGACCACTAGAGGGGG.

2.5. Lens epithelial cells differentiation from iPSC

Ten to fifteen stem cell-like clones (iPSC) were obtained per 10^5 mesenchymal cells, and transformed into lens epithelial cells using the following three-steps of Qui et al. [23] method: (i) Nogin (100 ng/ml) for 5 days, (ii) basic fibroblast factor (bFGF, 100 ng/ml), and bone morphogenetic protein-4 (BMP4, 20 ng/ml) from day 5 to day 13, (iii) Fibroblast growth factor 2 (100 ng/ml) and Wnt (20 ng/ml) from day 15 to day 30. The culture medium was refreshed every two days as described above.

3. Results

Fig. 1A and B shows the phase contrast images of mouse lens epithelial cells, and mesenchymal cells (following epithelial cells transition after 5-days), respectively. At day 5, the cell morphology became more irregular, elongated and fibroblast-like. After cells reached confluence, they were reprogramed to iPSC through viral transduction after 30 days (Fig. 1C). The lower panels of Fig. 1 show immunocytochemical expression of αB-crystallin in green by lens epithelial cells (Fig. 1D), whereas the mesenchymal cells lacked the expression of E-cadherin (in red), but exhibited a robust expression of alpha-smooth muscle actin (αSMA, in green) [Fig. 1E]. We also analyzed the expression levels of N-cadherin, which is also another mesenchymal marker (Supplemental

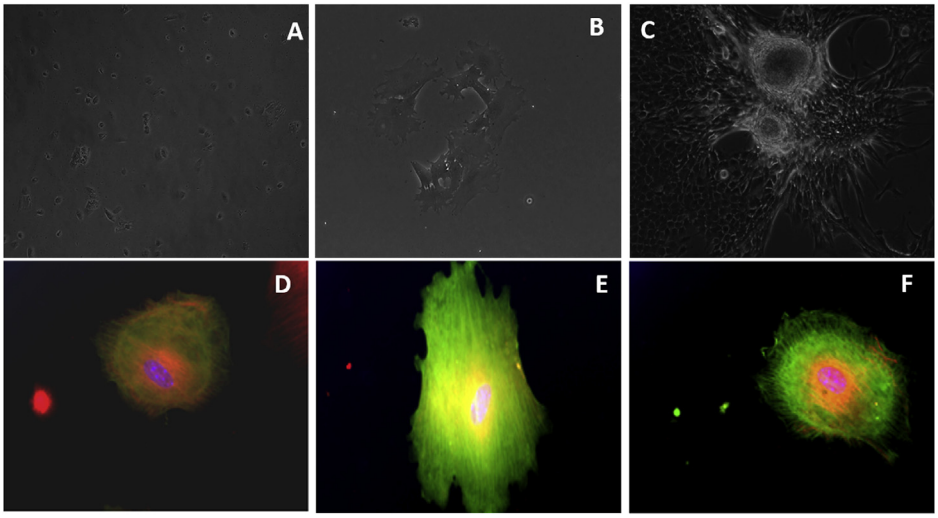

Fig. 1. A: Phase contrast image of lens epithelial cells that were allowed to grow for up to 72 h. B: Phase contrast image of lens Mesenchymal cells. C: Phase contrast image of lens induced pluripotent stem cells. Lower Panel: Immunocytochemistry of mesenchymal cells using different antibodies. (D): αB-Crystalline (green), (E): α-Smooth muscle actin (green) and E-Cadherin (red), and (F) Vimentin (green) and Connexin-43 (red). The cells were viewed by a Zeiss Axioplan 2 fluorescent microscope equipped with a CCD camera. (G): The fluorescent images D, E and F quantified using Image J. {(Sale Bar Fig A & C are 200 μM and 10× magnification), B,D,E & F are 100 μM and 20× magnification}.

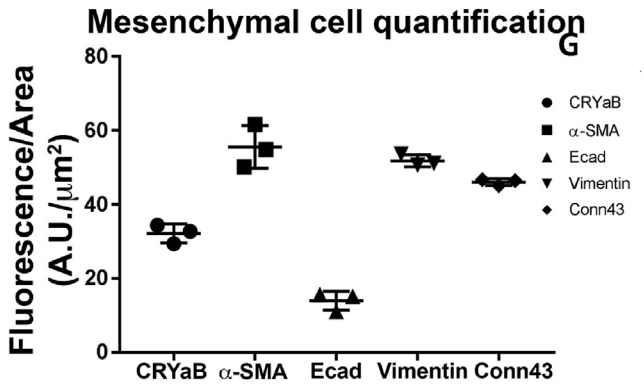

Fig. 1C). The N-cadherin expression was seen at the site of cell-cell contact. These cells also expressed connexin-43 (in red) and vimentin (in green) [Fig. 1F]. In these cells, DAPI showed the nuclear stain. The immunofluorescence images D, E and F was quantified using Image J and are shown in Fig. 1G.

Fig. 2 shows immunocytochemical analysis of the expression of mesenchymal markers (α-SMA), epithelial marker (E-cadherin), and iPSC markers (OCT4 and SOX1) at different time intervals. DAPI was used a nuclear stain and images shown here are co-localized images but DAPI was not used for expression analysis. The expression of α-SMA was barely detected at Day1 (Fig. 2A) and expression level increases at Day 5 (Fig. 2B) and the control which is mesenchymal cell (Fig. 2C) shows increased expression compared to Day 5. These fluorescent images were quantified (Fig. 2Q) using Image J as described in the Method section. Although all the cells showed the expression of α-SMA, but only the mesenchymal cells exhibited the characteristic filamentous architecture with well-formed fibers. Whereas epithelial marker was at the highest in cells at Day1 (Fig. 2E) and was minimal at Day 5 (Fig. 2F). The control cells did not express epithelial markers as shown in Fig. 2G and also very little expression in iPSC (Fig. 2H). The E cadherin fluorescence were also quantified using Image J as shown in Fig. 2R. The stem cell markers showed highest expression in iPS clones as shown in Fig. 2L and P. The stem cell markers were not expressed at the Day 1 (Fig. 2I and M), Day 5 (Fig. 2J and N) and also control (Fig. 2K and O).

Next, we analyzed the mRNA expression levels of marker proteins of epithelial, mesenchymal cells and iPSC using Q-RT PCR (Fig. 3A). Although previously it was shown that lens epithelial cells express mainly αA- and αB-crystallins whereas lens fibers express β- and γ-crystallins [27], later it was discovered that mRNA of βB1 and γS-crystallins also existed in the epithelial cells [28]. On the analysis, αB-crystallin transcript was expressed at high levels at day 0 in epithelial cells, but its expression was reduced to almost half in mesenchymal cells at day 5, and about 1/3 in iPSC (Fig. 3A). Similarly, the mRNA levels of E-cadherin was expressed at high levels in epithelial cells at day 0 but was reduced about 60% at day 5 in mesenchymal cells, and was further reduced in iPSC. This concurs with the earlier published results that cells lose their junctions made of E-cadherin following EMT transitions (see Introduction). Among the three connexins (Cx43, Cx46 and Cx50) that are expressed in the ocular lens, connexins Cx43 and Cx50 are the predominantly expressed in the epithelial cells [29]. The q-RT PCR analysis showed that the connexin 43 mRNA was at the highest levels in the epithelial cells on day 0, and its levels decreased by 75% in the mesenchymal cells and iPSC. Although not much difference in the α-SMA expression in epithelial/mesenchymal cells and iPSC was observed, the distinct expression differences were seen during immunocytochemical analysis as shown in Fig. 2A, B, C and D. Vimentin plays a central regulating repair function during healing of the lens epithelium following cataract surgery [30]. Vimentin mRNA also exhibited variable expression levels during different stages, i.e., it was highest in epithelial cells (on day 0) but was reduced to about 50% in mesenchymal cells on day 5, and also in iPSC. The mRNA levels of two iPSC markers (Sox1 and Oct 4) showed an increase in iPSC compared to in mesenchymal cells.

The epithelial cells that were *trans*-differentiated from iPSC were also immunocytochemically analyzed (Fig. 3B–E). A previous study has shown that the expression of p57KIP2 mRNA throughout murine lens morphogenesis and growth correlates with lens cell withdrawal from the cell cycle (indicated by changing patterns of BrdU incorporation), and the onset of lens fiber cell differentiation (demonstrated by β-

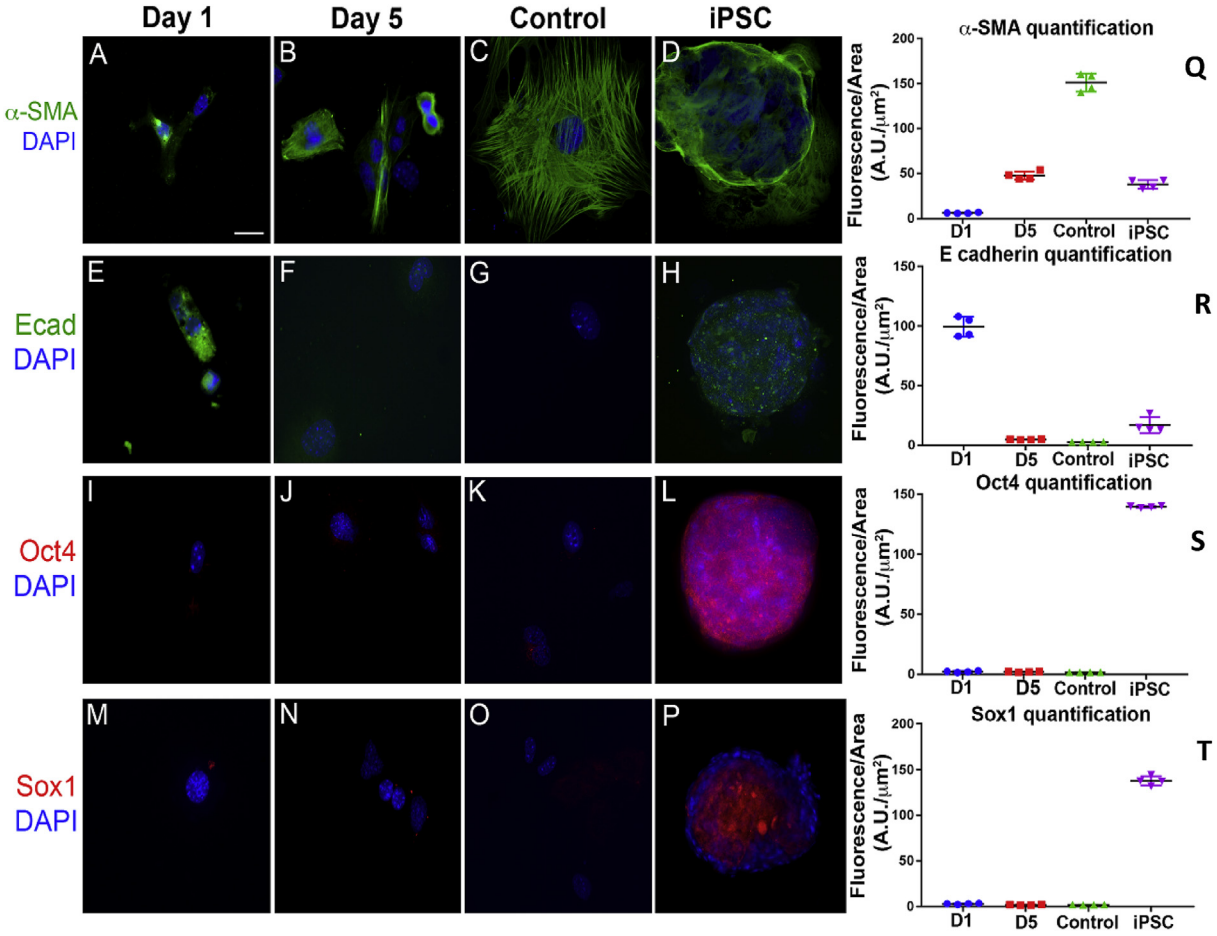

Fig. 2. Expression of epithelial (Ecadherin) and Mesenchymal (α-SMA) and stem cell markers (Oct4 and Sox1) at different stages. DAPI was used as a nuclear stain. Expression of mesenchymal marker (α-SMA and nuclear stain, DAPI) A (Day 1), B (Day 5), C (Control) and D (iPSC). The fluorescent images were quantified and is shown in Figure Q. Expression of Epithelial markers (ECad and nuclear stain) E (Day1), F (Day 5), G (control) and H (iPSC). The fluorescent images (E cadherin) were quantified and is shown in Figure R. Expression of stem cell marker (Oct4 and nuclear Stain) I (Day1), J (Day 5) K (control) and L (iPSC). The fluorescent images were quantified and is shown in Figure S. Expression of stem cell marker (Sox1 and nuclear Stain) M (Day1), N (Day 5) O (control) and P (iPSC). The fluorescent images were quantified and is shown in Figure T. (Scale Bar are 100 μM and 20× magnification).

crystallin expression) [31]. The report showed that at E11.5, posterior lens vesicle cells begin to express p57 mRNA and exit from the cell cycle before their elongation and subsequent expression of β-crystallin. Our differentiated iPSC also showed expression of p57KIP2 (Fig. 3B) along with the expression of αA-crystallin. The differentiated cells also expressed vimentin and connexins 54 (Fig. 3C), and α-SMA, E-cadherin and βA3/A1-crystallin as shown in Fig. 3C, D and 3(E), respectively. The fluorescent images C, D and E were quantified by Image J and is shown in Fig. 3G. Fig. 3F shows the phase contrast images of the differentiated epithelial cells derived from iPSC.

4. Discussion

Although cataract affects 20 million people worldwide and causes 51% of world's blindness [32], only a few investigators have used stem cell therapy and drug screening to cure different types of cataract. The aims of some of these studies were to elucidate factors regulating lens development, and drug screening to cure/delay the different types of cataracts. These include lens regeneration from progenitor cells as lentoid bodies using either human embryonic stem cells [21], human iPSC [22], or lenses of cataract patients [23]. A gene therapy approach has been suggested for PCO to eradicate post-cataract surgery-proliferating epithelial cells by introducing a cytotoxic gene in the capsular bag while respecting the surrounding ocular tissues [16]. Further, researchers have reported that lanosterol as a critical molecule in the prevention of lens protein aggregation, and pointed to a novel strategy for cataract prevention and treatment [33].

Pluripotency in induced pluripotent stem cells (iPSC) has been achieved by expressing ectopic factors (OCT-4, SOX-2, c-Myc, and KLF-4) that are highly expressed in embryonic stem cells, and are used to reset epigenetic and transcriptional status of somatic cells to those of pluripotent cells [34]. The pluripotency of iPSC represents the unique ability to form any specialized and differentiated cell type of the organism from which they are derived. This has enormous potential allowing for the generation of disease-specific pluripotent cells (e.g., iPSCs from a patient with diabetic cataract) that could be used to study both lens development and mechanism of cataractogenesis. In the regenerative medicine, the earlier view was to repair or replace cells that are damaged due to a disease or injury by iPSC. It is now expanded to include disease-specific iPSC to test new candidate drugs, which previously relied on the animal models.

We used mouse lens epithelial cells-derived mesenchymal cells to successfully reprogram to IPSc by delivering the reprogramming factors twice within 24 h as described previously [25]. Next, the iPSCs were incubated with growth factors in three consecutive steps to generate lens epithelial cells [23]. At various time points, the cells/clones expressed epithelial markers (Connexin-43 and E-cadherin), mesenchymal markers (Alpha-smooth muscle actin), and lens-specific markers (CRYAB) and stem cell markers (Sox1, Oct4, SSEA4 and Tra60) [Figs. 1 and 2]. Similarly, the results of Fig. 3 showed that iPSC-derived

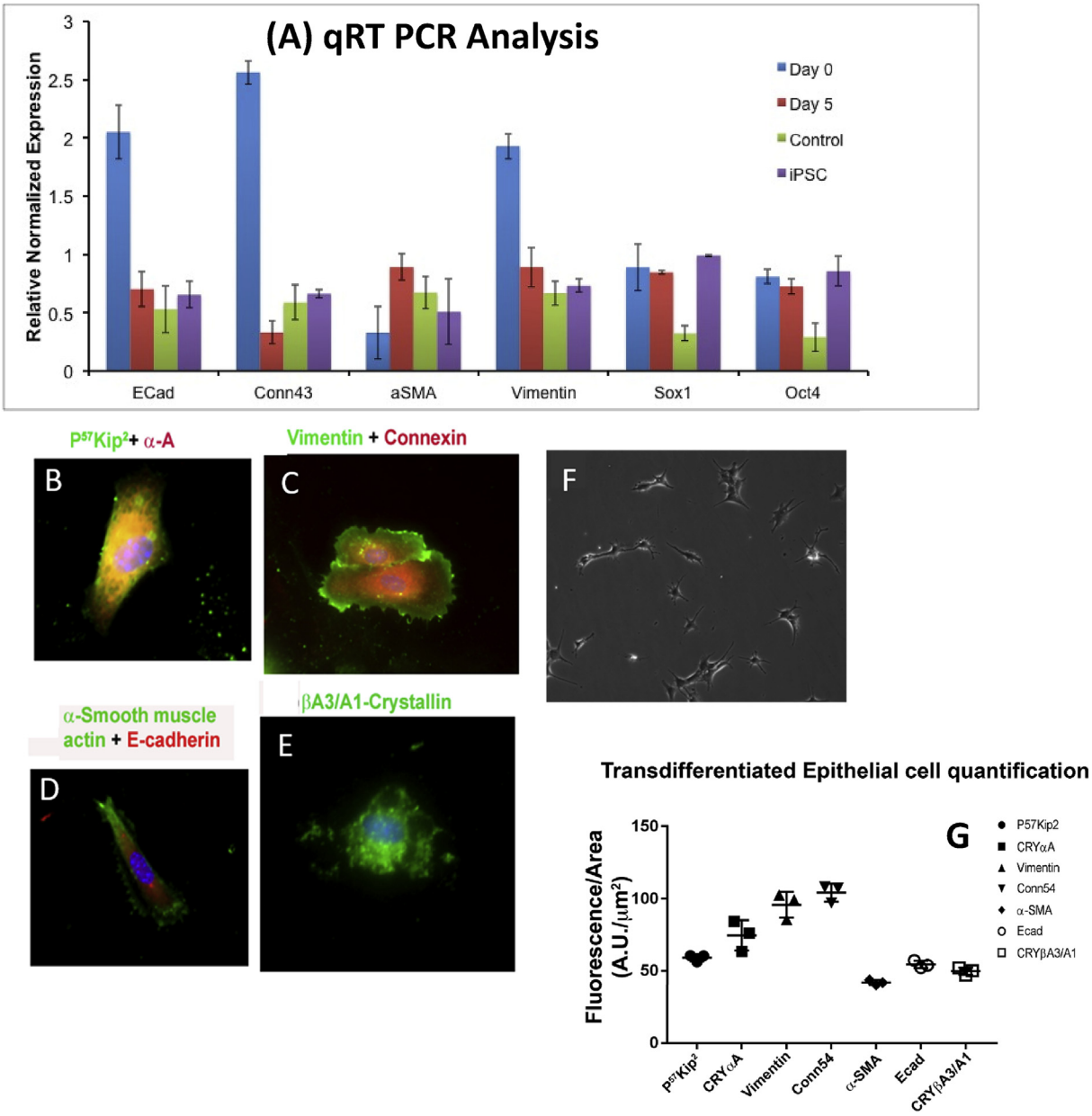

Fig. 3. qRT-PCR expression levels of epithelial and Mesenchymal and stem cell markers at different stages. Fig. 3: B–D: Immunocytochemistry of lens specific markers in differentiated cells. (B): P^{57}Kip (green) and αA-Crystallin (red), (C): Vimentin (green) and Connexin (red) (D): α-Smooth muscle actin (green) and E-Cadherin (red), and (E): βA3/A1-Crystallin (green). F: Phase contrast image of differentiated cells. The fluorescent images were quantified and is shown in Figure G. (Scale Bar Fig. B,C,D,E are 100 μM and 20× magnification) and F are 200 μM and 10× magnification.

epithelial cells also showed the epithelial cells markers such as αA- and βA3/A1-crystallins, vimentin, and E-cadherin.

The α-SMA is a marker for epithelial-mesenchymal transition (EMT) during PCO where the remnant epithelial cells transition to fibrotic-mesenchymal cells. Lens epithelial cells also express α-SMA [35] and our results show that it is also expressed by the lens stem cells. Vimentin is also constitutively expressed by lens epithelial cells, and plays a critical role in repair and movement of the cells in response to wounding [30]. Cadherins (E-, N-, P- and R-cadherin), a family of calcium-dependent cell adhesion molecules, share a high degree of structural similarity which undergo homophilic interactions to maintain cell-cell contacts at adherens junctions [36]. It has been shown in embryonic stages of double mutant mice that E− and N-cadherin play an essential role during lens vesicle separation [37] Our results support earlier results that E-cadherin plays a critical role in the lens development and that it lost after 5 days in wild type mice [38]. N-cadherin which is a marker for mesenchymal cells was present at the site of cell-cell contact. Those cells that don't have contacts with another cells didnot express N-cadherins (Supplemental Fig. 1C). But these cells expressed vimentin which is another mesenchymal marker that is widely used (Supplemental Fig. 1D). We have also shown that our differentiated cells express p57KIP2, which is a marker for fiber cell differentiation.

Our study will provide an opportunity to gain knowledge regarding regulatory elements during the EMT transition by differentiating iPSC-derived epithelial cells to mesenchymal cells. Therefore, the lens iPSC model could be a valuable tool to study lens differentiation, and also could be used as a model to study EMT transition during PCO development.

Grant information

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Transparency document

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Appendix A. Supplementary data

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