

# Myh11 Lineage Corneal Endothelial Cells and ASCs Populate Corneal Endothelium

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**PURPOSE.** To establish Myh11 as a marker of a subset of corneal endothelial cells (CECs), and to demonstrate the feasibility of restoring the corneal endothelium with *Myb11*-lineage (*Myb11*-Lin[+]) adipose-derived stromal cells (ASCs).

**METHODS.** Intraperitoneal administration of tamoxifen and (Z)-4-hydroxytamoxifen eyedrops were used to trace the lineage of Myh11-expressing cells with the *Myb11*-Cre-ER<sup>T2</sup>-floxedTomato mouse model. Immunostaining and Western blot characterized marker expression and spatial distribution of *Myb11*-Lin(+) cells in the cornea, and administration of 5-ethynyl-2'-deoxyuridine labeled proliferating cells. ASCs were isolated from epididymal adipose Myh11+ mural cells and treated with cornea differentiation media to evaluate corneal endothelial differentiation potential. Differentiated ASCs were injected into the anterior chamber to test for incorporation into corneal endothelium following scratch injury.

**RESULTS.** A subset of CECs express Myh11, a marker previously thought restricted to only mural cells. *Myb11*-Lin(+) CECs marked a stable subpopulation of cells in the corneal endothelium. *Myb11*-Lin(+) ASCs undergo CEC differentiation in vitro and incorporate into injured corneal endothelium.

**CONCLUSIONS.** Dystrophy and dysfunction of the corneal endothelium accounts for almost half of all corneal transplants, the maintenance of the cornea endothelium is poorly understood, and there are a lack of mouse models to study specific CEC populations. We establish a mouse model that can trace the cell fate of a subpopulation of CECs based on Myh11 expression. A subset of ASCs that share this Myh11 transcriptional lineage are capable of differentiating into CECs that can incorporate into injured corneal endothelium, revealing a potential cell source for creating engineered transplant material.

Keywords: cornea endothelium, adipose-derived stromal cell, cornea, endothelium, myh11

The cornea endothelial cell (CEC) layer resides in the innermost portion of the cornea and composed of a single layer of hexagonal cells connected by tight junctions.<sup>1</sup> CECs serve a critical role in maintaining hydration and exchanging nutrients between the anterior chamber and the outer layers of the cornea, including the outermost epithelium and intermediate stromal layer.<sup>2,3</sup> Active transport of ions across the endothelium, paired with its semipermeable membrane, leads to the “pump-leak” mechanism,<sup>2</sup> where water is constantly cycled through the corneal cell layers. This cycling maintains optical transparency for the tissue,<sup>4</sup> allowing light to pass through unimpeded to the retina and facilitates sight. With the absence of a blood vessel network in the cornea to provide metabolic support, the constant fluid cycling directed by the cornea endothelium is also responsible for delivering necessary nutrients to the outer cornea layers.<sup>2</sup>

Proper function of the corneal endothelium is critical for the homeostatic maintenance and function of the tissue, with deficient fluid flux leading to tissue dehydration and inadequate nutrient supply, whereas excess fluid flux leads to corneal edema and a reduction in visual acuity. To preserve function, CEC cell populations must remain at sufficient densities to

maintain the basement membrane and outer tissue layers. However, previous research suggests that the CEC layer cannot be regenerated or repopulated in adult tissue, and CECs dropout with aging, injury, surgical trauma, and dysfunction,<sup>5</sup> often requiring cornea transplant surgery.<sup>6</sup>

Disruption of this cell population can occur with various corneal diseases,<sup>7</sup> hypoxia caused by long-term wear of contacts,<sup>8</sup> and cornea transplant surgery.<sup>9</sup> Paradoxically, corneal transplants are often the only therapeutic option for a depleted corneal endothelium, but the trauma from the surgical procedure can lead to further dropout of CECs.<sup>10</sup> Transplant patients exhibit a 4-fold dropout of the CEC layer compared with uninjured corneas.<sup>11</sup> Without an endogenous cell population to repopulate this tissue layer, which is required for homeostatic maintenance of the cornea and critical for visual acuity, a clinically relevant exogenous cell population is needed to prevent attrition of this cell layer and preserve its function. Adipose-derived stromal cells (ASCs) represent a cell population that can be efficiently harvested in the clinic from readily available adipose tissue, cultured, and reintroduced into the patient as an autologous stem cell

therapy that avoids immune responses associated with use of exogenous tissue sources.<sup>12</sup>

We demonstrate that CECs share *Myb11* transcriptional lineage with a subpopulation of ASCs, a marker previously associated only with vascular smooth muscle cells<sup>13</sup> and pericytes,<sup>14</sup> using a lineage tracing mouse model and confirmed with antibody immunofluorescence and Western blot. Informed by their shared transcriptional lineage, we demonstrate that cultured ASCs can differentiate into a phenotype associated with CECs and are capable of adhering to injured adult corneal endothelium when exogenously injected into the anterior chamber after a scratch injury. Whole tissue image analysis of corneas from *Myb11* lineage tracing mice indicates that Myh11 exclusively marks a stable subpopulation of CECs and cells that express Myh11 may serve some unknown function in maintenance of the endothelium. We provide the first lineage tracing mouse model for selectively following a subset of endothelial cells in the cornea that can trace their cell fate in injury and disease, and demonstrate the potential to supplement the corneal endothelium with a clinically relevant cell source.

## METHODS

### Animals

All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We generated *Myb11-CreER<sup>T2</sup>* mice that were crossed with ROSA26-STOP<sup>FLOX</sup>eYFP<sup>+/+</sup> (stock number 006148; The Jackson Laboratory, Bar Harbor, ME, USA) and ROSA26-STOP<sup>FLOX</sup>tdTomato<sup>+/+</sup> (stock number 007914; The Jackson Laboratory) to generate *Myb11-CreER<sup>T2</sup>; ROSA26-STOP<sup>FLOX</sup>eYFP<sup>+/+</sup>* (*Myb11-eYFP*) and *Myb11-CreER<sup>T2</sup>; ROSA26-STOP<sup>FLOX</sup>tdTomato<sup>+/+</sup>* (*Myb11-RFP*) mice. Mice were treated with tamoxifen as done previously (Supplementary Methods S1: Tamoxifen Treatment).

### Immunohistochemistry and Immunocytochemistry

Immunostaining was carried out as done previously<sup>15</sup> (Supplementary Methods S2: Immunofluorescence).

### 5-Ethynyl-2'-Deoxyuridine (EdU) Assay

To label proliferating cells in the cornea, we used 5-ethynyl-2'-deoxyuridine (EdU) (A10044; ThermoFisher, Waltham, MA, USA) and Click-iT EdU AlexaFluor 647 Imaging Kit (C10340; ThermoFisher). Tamoxifen-induced *Myb11-RFP* adult male mice were intraperitoneally injected with 200 mg/kg EdU at 12 weeks of age to examine cell proliferation, with only a single injection used because higher concentrations can exhibit toxic effects.<sup>16</sup> Thirty days after injection, corneas were harvested and fixed in 3.7% paraformaldehyde for 15 minutes at room temperature. Following fixation, corneas were washed with 3% BSA in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 20 minutes at room temperature. The tissue was washed in 3% BSA in PBS. Corneas were incubated with Click-iT reaction cocktail (containing reaction buffer, CuSO<sub>4</sub>, Alexa Fluor 647 Azide, and reaction buffer additive) for 30 minutes at room temperature. Last, tissues were washed with 3% BSA for 30 minutes twice, and then stained for 4',6-diamidino-2-phenylindole (DAPI) and whole-mounted for confocal imaging.

## Primary Cell Culture

*Myb11-Lin(+)* mural cells from the epididymal adipose tissue was harvested for collection and culturing by previous a previous protocol<sup>17</sup> (Supplementary Methods S3: Primary Cell Culture).

## In Vitro CEC Differentiation

*Myb11-Lin(+)* ASCs were uplifted and cultured on FNC Coating Mix (NC9971265; ThermoFisher). Once *Myb11-Lin(+)* ASCs reached greater than 90% confluency, the standard media was replaced by corneal endothelial differentiation media as previously described.<sup>18</sup> Briefly, *Myb11-Lin(+)* ASCs were exposed for 3 days to dual Smad induction media, which consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% KnockOut serum replacement (10828028; ThermoFisher), 1% nonessential amino acids (11140076; ThermoFisher), 500 ng/mL Noggin (SRP3227; Sigma, St. Louis, MO, USA) 10 μM SB431542 (S4317; Sigma), 1 mM L-glutamine (25030081; ThermoFisher), 0.1 mM betamercaptoethanol (21985023; ThermoFisher), and 8 ng/mL recombinant murine FGF-2 (450-33; PeproTech, Rocky Hill, NJ, USA). On day 3, the dual Smad induction media was replaced with dual Smad induction media supplemented with 0.1X B27 supplement (17504044; ThermoFisher), 10 ng/mL recombinant mouse Dkk-2 (2435-DKB/CF; R&D Systems, Minneapolis, MN, USA), and 10 ng/mL recombinant murine PDGF-BB (315-18; PeproTech). This media solution was changed every 3 days over the course of 14 days. After the course of 14 days, cells were fixed and stained using methods described above.

## CEC In Vivo Injury Model

We adopted a previous animal procedure to introduce injury to the CEC layer, followed by local delivery of *Myb11-Lin(+)* ASCs cultured in CEC-differentiated media for 14 days.<sup>19</sup> Briefly, adult C57Bl/6j mice were placed under isoflurane anesthesia, and proparacaine hydrochloride ophthalmic solution was applied to the eyes as a topical anesthetic. Injury and injections were done on the right eye due to difficulty of angle and manipulation with one master-hand, and separate mice were given injury with injection of vehicle control. A small entry in the peripheral cornea was introduced with a 33-gauge needle. To induce injury and removal of CECs, a 36-gauge needle was used to scrape along the bottommost layer of the cornea, while avoiding the iris and lens. A total of 5000 *Myb11-Lin(+)* ASCs cultured in CEC differentiation media were delivered in 3 μL of DMEM media supplemented with 100 μM Rho Kinase (ROCK) inhibitor Y-27632 (Y0503; Sigma) and 100 μM TGFβR inhibitor SB4314542 (S4317; Sigma). After the delivery of cells into the aqueous humor, the mice were immediately overturned on their backs tilted to lower the injected eye and allow the injected cells to settle toward the CEC layer and adhere. The mice were kept in isoflurane anesthesia for 3 hours to allow for cells to adhere to the CEC layer. Seven days after delivery of *Myb11-CEC*-differentiated cells, corneas were harvested and immunostained as described above.

## Immunoblotting

Immunoblotting was performed as previously described<sup>20</sup> (Supplementary Methods S4: Immunoblotting).

## Image Analysis and Statistical Analysis

All data were processed using MATLAB (MathWorks, Natick, MA, USA) and ImageJ (<http://imagej.nih.gov/ij/>; provided in the

public domain by the National Institutes of Health, Bethesda, MD, USA). Please see Supplementary Methods S5 and S6 for details. For plots, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . Source code and data available at: [https://github.com/uva-peirce-cottler-lab/cornea\\_endothelial\\_public](https://github.com/uva-peirce-cottler-lab/cornea_endothelial_public).

## RESULTS

### Myh11-Lin(+) Cells Are Exclusively Detected in the CEC Layer

Male *Myb11*-Cre-ER<sup>T2</sup>-flox-tdTomato mice were administered 2 weeks of intraperitoneal tamoxifen followed by a 4-week chase period, resulting in the labeling of Myh11 expressing cells with an RFP lineage marker. Myh11 expression has been claimed to be limited to only mural cells, and RFP+ cells were found as expected along the vasculature (not shown). However, examination of the cornea revealed quite unexpectedly RFP+ cells throughout the cornea (Fig. 1A). Male mice administered vehicle control did not show any RFP-expressing cells in the cornea, demonstrating that RFP expression was not due to leaky cre-recombinase with the mouse model, but rather specifically resulted from tamoxifen induction (Supplementary Fig. S1A). RFP+ cells, albeit at a lower cell density, were also observed in the cornea with 2 days of local administration of eyedrops containing 4-hydroxytamoxifen with 21 days of chase, suggesting these RFP+ cells were labeled locally and did not originate from the circulatory system (Supplementary Figs. S1B, S1C).

Confocal imaging of sagittally sectioned cornea layers revealed that these RFP-expressing cells were localized to the corneal endothelium (Fig. 1B). A high-resolution confocal z-stack visualized with a lateral projection of cornea (Fig. 1C), three-dimensional segmentation (Figs. 1D–G), and tissue layer-specific coronal z-projections (Figs. 1H–J) confirmed that RFP expression was exclusively found within the corneal endothelial layer. DAPI labeling confirmed confinement of RFP cells within a homogeneously spaced and highly organized cobblestone arrangement of cell nuclei, consistent with corneal endothelium.

### Myh11-Lin(+) Cells in the Cornea Express Myh11 and Canonical CEC Markers

After establishing the existence of *Myb11*-lineage cells (*Myb11*-Lin(+)) in the CEC layer of adult *Myb11*-RFP mice, immunolabeling was used to characterize canonical CEC marker expression of *Myb11*-Lin(+) corneal cells and determine if Myh11 protein was actively produced and not merely the *Myb11* transcript. Immunofluorescence revealed Myh11 expression not only in smooth muscle cells and pericytes along corneal limbal vessels, but also cells in the avascular CEC layer (Figs. 2A, 2B).

Expression of Myh11 protein in the cornea was confirmed with surgical isolation of avascular cornea from the vascularized limbal vessels and sclera through immunoblotting for Myh11 and CD31, a vascular endothelial cell marker. As expected with vascularized tissue, samples from sclera had detectable levels of Myh11 and CD31 (Fig. 2C). In contrast, samples isolated from cornea lacked CD31 expression, because no blood vessels exist within corneal tissue (Fig. 2D,  $t$ -test,  $P = 0.0062$ ); however, corneal samples exhibited Myh11 expression at levels comparable to those found in the sclera (Fig. 2E,  $t$ -test,  $P = 0.357$ ). Corneal *Myb11*-Lin(+) cells, labeled with RFP, were confirmed to exhibit the same marker expression as CECs,<sup>21</sup> being positively marked with the junctional proteins N-cadherin and ZO-1, while lacking expression of CD31, CD34, and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) (Figs. 2F–H). The lack of

vascular (CD31, CD34) and perivascular ( $\alpha$ SMA) markers lend further evidence that these corneal *Myb11*-Lin(+) cells are not affiliated with vascular cells and represent a distinct cell type from pericytes and smooth muscle cells.

### Myh11-Lin(+) CECs Do Not Migrate Into the Central CEC Layer

Although the *Myb11*-Lin(+) cells in the CEC layer express Myh11 transcript and protein, and exhibit a CEC phenotype, there remains the possibility that a portion of these cells are perivascular in origin, having migrated from limbal vessels and scleral vasculature. If these cells are indeed migrating from the peripheral cornea, *Myb11*-Lin(+) cell density should change over time as more induced RFP+ cells migrate from peripheral to central cornea. At early timepoints, RFP+ cell density should be higher in the peripheral cornea compared with central, showing a negative slope in radial density of RFP+ cells toward the center of the cornea. We examined multiple timepoints following acute tamoxifen induction using eyedrops or intraperitoneal injection. High-resolution images of the entire cornea were automatically segmented to separate out nuclei of the CEC layer and determine CEC expression of RFP (Supplementary Fig. S2).

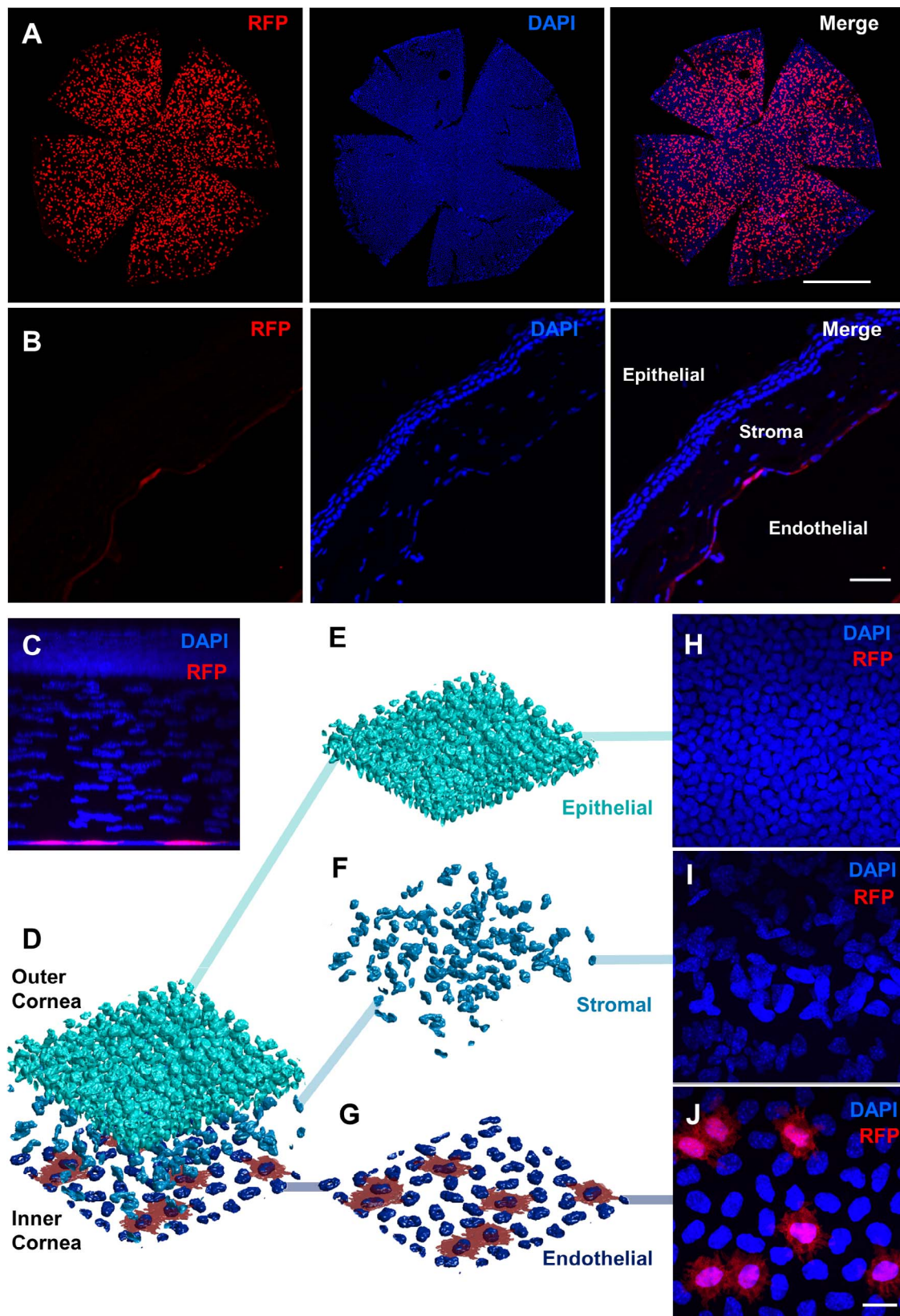
Tamoxifen eyedrops were administered over a 12-hour period followed by a short-term 12-hour chase period. Corneas were then harvested and RFP+ cell location was quantified relative to radial distance from the corneal center (radial densities were normalized to area of each bin). A positive slope was found across the 95% confidence interval, strongly suggesting that *Myb11*-Lin(+) cells did not migrate from the edge of the cornea (radial value of 0) to its center (radial value of 1) (Supplementary Fig. S3, 95% confidence interval of slope in brackets with best fit of linear model). Eye drop induction for 3 days with a 2-day or 21-day chase revealed no difference in the total number of *Myb11*-Lin(+) cells (Fig. 3A,  $t$ -test,  $P = 0.411$ ). Both timepoints showed a slightly positive slope using a linear model mapping the fraction of RFP+ CECs to the radial distance from the peripheral cornea (Figs. 3B–E).

The same trends were observed in lineage-traced mice treated with 2 weeks of intraperitoneal injections of tamoxifen at 6 weeks and 16 weeks of age, both with 4 weeks of chase time after induction. There was no change in total number of *Myb11*-Lin(+) cells, and both timepoints had a positive relation between the fraction of marked cells and radial distance from center of the cornea (Figs. 4A–E). These findings across multiple timepoints strongly suggest RFP+ CEC distribution does not vary according to time from induction. There was an estimated 10-fold difference between RFP+ cell density between eyedrops of 4-hydroxytamoxifen and intraperitoneal injection of tamoxifen, but we attribute this to differences in the efficacy and duration of tamoxifen delivery.

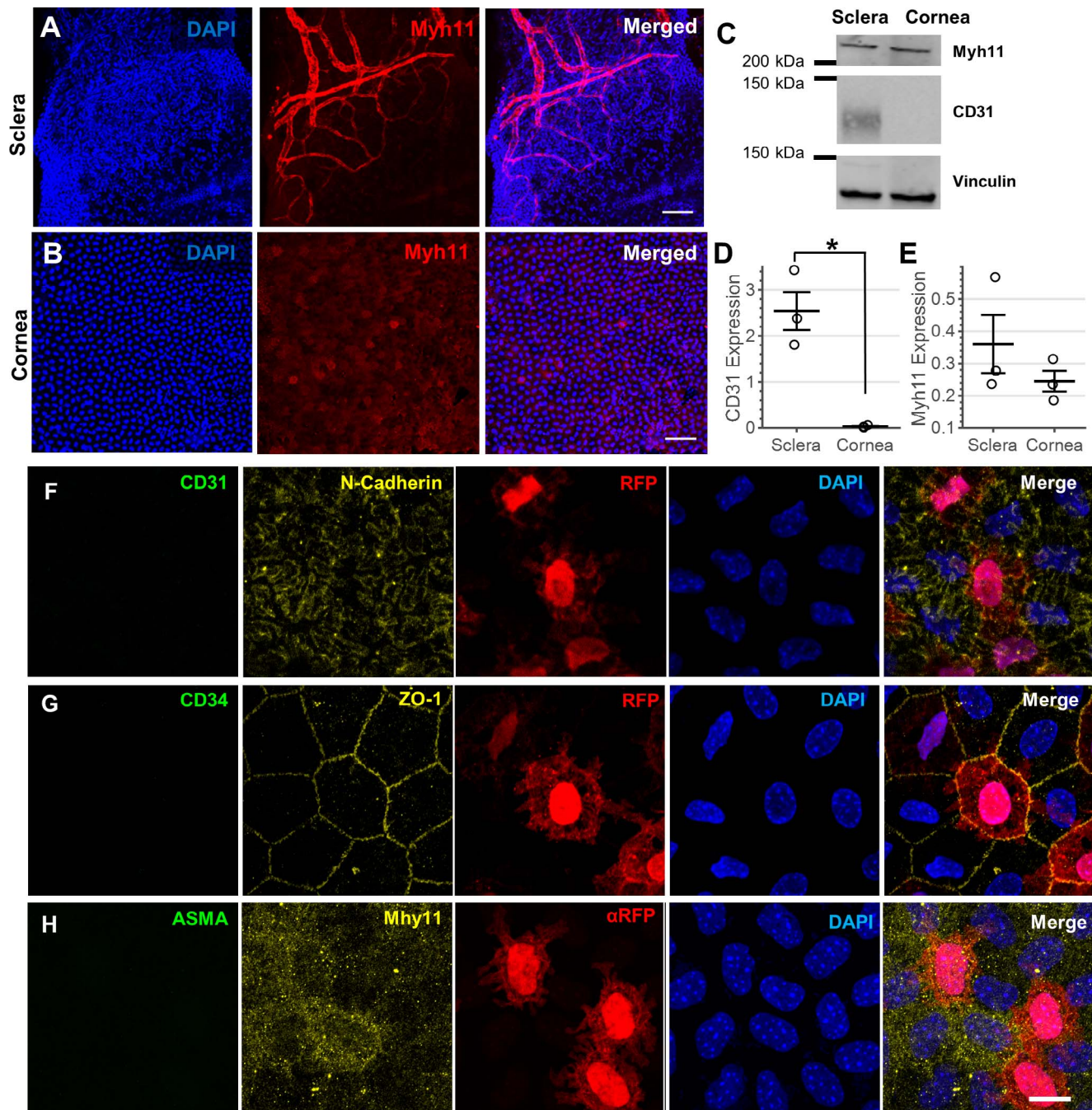
### Myh11-Lin(+) CECs are Slow-Cycling or Nonproliferative

There is sparse evidence to suggest that CECs proliferate in vivo, with one report suggesting that human adult corneas may contain proliferating CECs in the extreme periphery of the cornea. Importantly, this study was done using an explant model, which may present unique environmental conditions and cell behaviors as compared with in vivo.<sup>22</sup> Adult tamoxifen-induced *Myb11*-RFP mice were intraperitoneally injected with 200 mg/kg of EdU, and after 30 days, we harvested the cornea tissue to investigate the proliferation of *Myb11*-Lin(+) CECs. As expected, EdU was detected in the proliferating corneal epithelial cells<sup>23</sup>; however, we did not find EdU labeling in *Myb11*-Lin(+) CECs or any other RFP-CECs





**FIGURE 1.** Myh11 lineage tracing marks a subpopulation of cells on the basal surface of the cornea. (A) Confocal tile of the cornea of *Myh11*-Lin(+) male mice treated with 2 weeks of tamoxifen intraperitoneal injections with a 4-week chase, showing RFP lineage-labeled cells (red) and DAPI-labeled cell nuclei (blue). Scale bar: 1 mm. (B) Sagittal cryosection of cornea including the epithelial cell layer, stroma, and endothelial layer annotated from cell nuclei structure, with RFP-labeled lineage cells restricted to the endothelial layer. Scale bar: 50  $\mu$ m. (C) Lateral maximum intensity projection of high-resolution confocal z-stack through cornea. Scale bar: 25  $\mu$ m. (D) Three-dimensional reconstruction of cell nuclei, with clear separation of (E, H) epithelial, (F, I) stromal, and (G, J) endothelial cell layers delineated by cell nuclei structure and morphology. Scale bar: 25  $\mu$ m.



**FIGURE 2.** Myh11 protein is found in the avascular cornea, and Myh11 lineage cells of the cornea express markers for CECs. Immunostaining with anti-Myh11 antibody in the (A) sclera limbal vessels and (B) cornea endothelium (scale bar: 100  $\mu$ m). (C-E) Confirmation of Myh11 protein expression with Western blot of surgically isolated sclera and avascular cornea. (F) Immunostained fluorescent images of *Myh11-Lin(+)* cells in basal layer of cornea with anti-CD31 (green), anti-N-cadherin (yellow), anti-RFP (red), and DAPI (blue). (G) *Myh11-Lin(+)* RFP cells labeled with CD34 (green), ZO-1 (yellow). (H) *Myh11-Lin(+)* cells immunostained with anti- $\alpha$ SMA (green) and anti-Myh11 (yellow). Scale bar: 15  $\mu$ m.

(Supplementary Fig. S4). Unlike what has been observed in other preclinical models,<sup>24</sup> our results corroborate with the consensus finding that in adult murine tissue, healthy CECs are slow cycling or nonproliferating.<sup>25</sup>

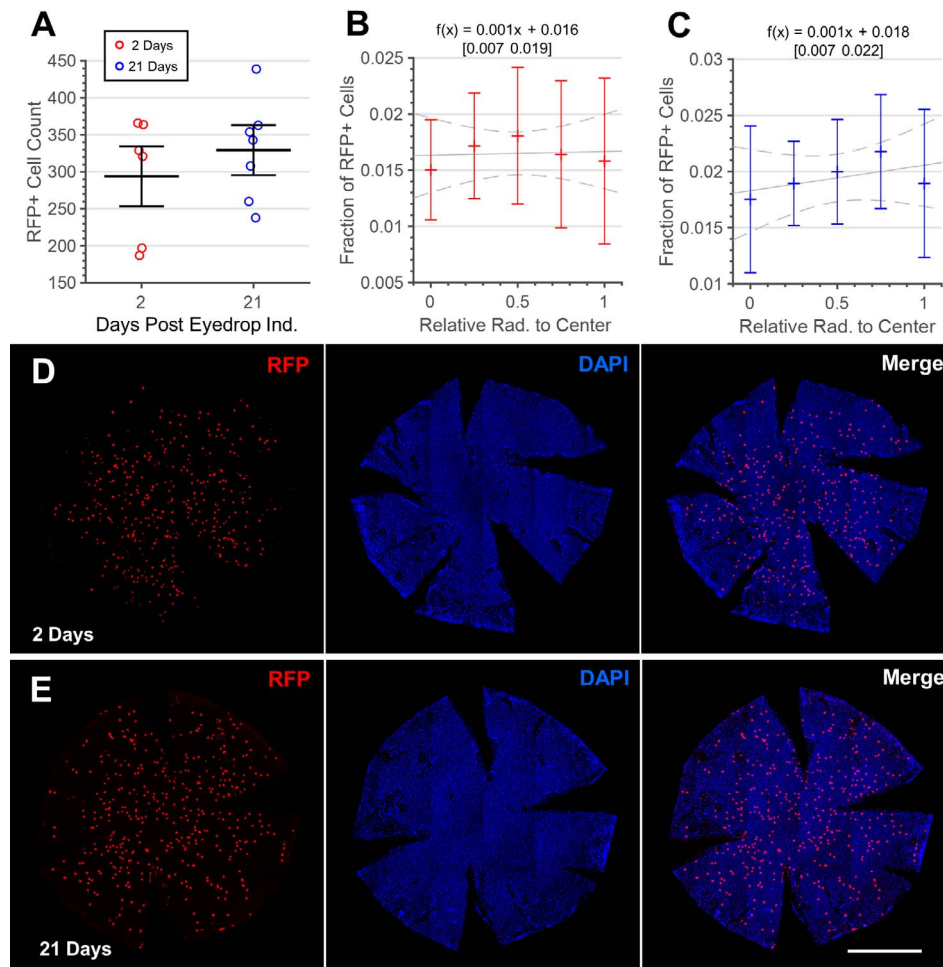
#### Cultured Myh11-Lin(+) ASCs Differentiate Into CEC-like Cells

Restoration of the corneal endothelium has previously been achieved by intraocular delivery of exogenous cells.<sup>26,27</sup>

Multipotent adult stromal cells may offer the potential for generating autologous CECs that would be resistant to rejection, and adipose tissue in particular offers a plentiful supply of such cells that can be easily harvested from patients. We explored the potential of using ASC-derived CECs to populate damaged endothelium.<sup>28</sup>

Fortuitously, Myh11+ mural cells are suggested to be putative ASCs, and it is possible that the shared transcriptional Myh11 lineage may be favorable in restoring the corneal endothelium. ASCs were isolated by collecting yellow fluorescent protein





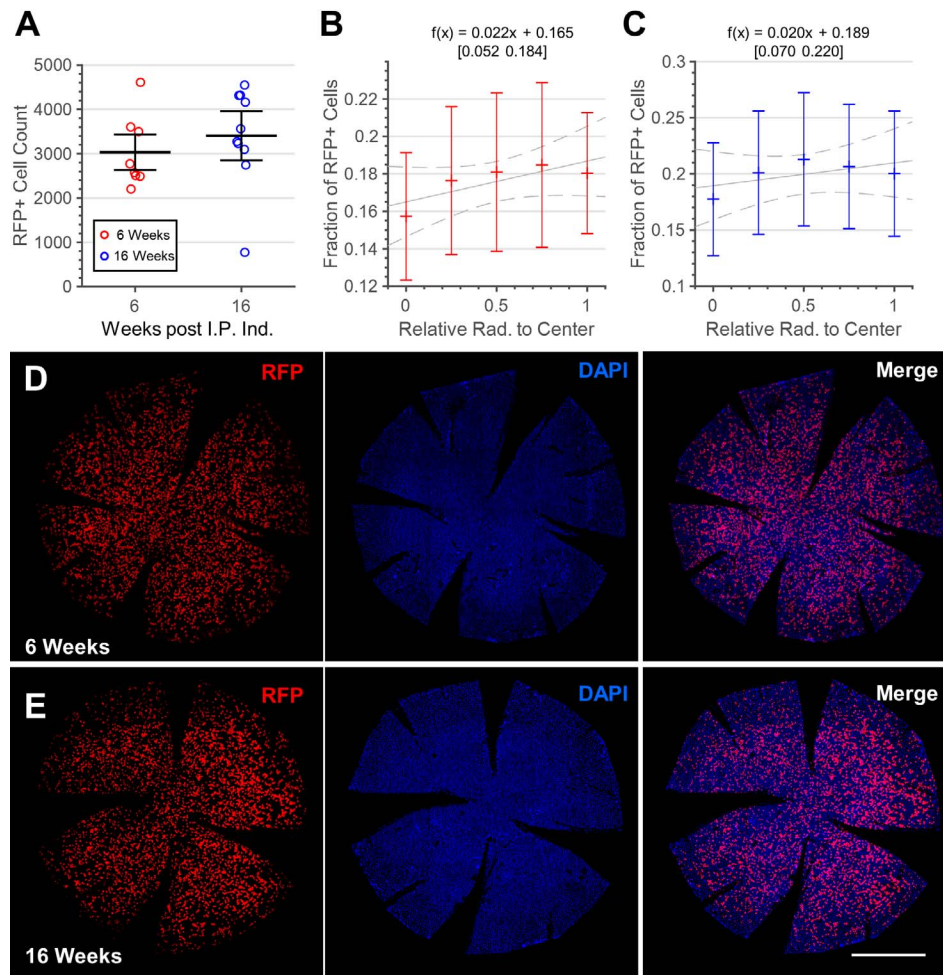
**FIGURE 3.** Myh11 lineage tracing from local eyedrop tamoxifen induction demonstrates no short-term peripheral to central corneal migration of labeled cells. (Z)-4-Hydroxytamoxifen eyedrops were used to induce RFP lineage marker in Myh11+ CECs. (A) Counts of *Myb11*-Lin(+) RFP-expressing cells in the cornea 2 and 21 days chase post-tamoxifen induction show no significant difference. Radial distribution of *Myb11*-Lin(+) cells from periphery (0) to center (1) of the cornea with (B) 2 days of chase and with (C) 21 days of chase do not show higher peripheral than central labeling, as would be expected if labeled cells were originating in the periphery and migrating centrally (95% confidence interval of slope in brackets). Representative images from (D) 2 days and (E) 21 days of chase post-tamoxifen induction with RFP (red) and DAPI (blue). Scale bar: 1 mm.

(YFP)-labeled *Myb11*-Lin(+) mural cells from the epididymal fat pad using enzymatic digestion, followed by fluorescence-activated cell sorting on YFP expression and immediate culturing of these sorted cells. Once near-confluent, YFP+ ASCs were cultured in a Smad inhibitor-based CEC differentiation media that has been previously used to induce CEC markers in human embryonic stem cells.<sup>18</sup> After culturing YFP+ ASCs for 3 days in CEC differentiation media treatment, Western blot analysis (Fig. 5A) indicated a reduction in expression of the fibroblast marker HSP90<sup>29</sup> (Fig. 5B, unpaired *t*-test,  $P = 0.0396$ ) and a slight trend of lower  $\alpha$ SMA expression (Fig. 5C, paired *t*-test,  $P = 0.298$ ). *Myb11*-Lin(+) derived ASCs did not initially express N-cadherin before treatment with CEC differentiation media (Fig. 5D), but they acquired expression after 14 days of treatment (Fig. 5E). During this time in culture, *Myb11*-Lin(+) derived ASCs underwent morphological changes from an elongated, spindle-shaped fibroblast shape to a smaller, round phenotype.

#### Local Delivery of Differentiated Myh11-Lin(+) ASCs Into Injured CEC Layer

We next tested whether CECs derived in vitro from *Myb11*-Lin(+) ASCs could be exogenously delivered intraocularly

and incorporated into the corneal endothelium. After introducing mechanical injury to the CEC layer of C57Bl/6J mice using an established protocol,<sup>19</sup> we injected 5000 CEC-differentiated *Myb11*-Lin(+) ASCs into the anterior chamber of the eye; 100  $\mu$ M of Rho Kinase (ROCK) inhibitor, Y-27632, and 100  $\mu$ M TGF $\beta$ R inhibitor, SB4314542, were coinjected to improve the engraftment of these exogenous cells.<sup>19</sup> Immediately after injection, mice were positioned so that the anterior segment of the eye faced downward to promote adherence of injected cells to the CEC layer. One week after local delivery of these *Myb11*-Lin(+) ASC-derived CECs, we examined adherence and incorporation of these cells to the corneal endothelium. YFP+ labeled cells were found adherent to the corneal endothelial cell layer at the site of the injury and continued to express N-cadherin (Fig. 5F), but incorporation overall was less than 1% of injected cells. The results demonstrate it is feasible for Myh11+ ASC-derived CECs to be targeted to the corneal endothelium, but low incorporation rates and potential phenotypic differences from surrounding endogenous CECs suggest additional refinement to technique is needed to establish this cell source as a viable therapeutic.



**FIGURE 4.** Myh11 lineage tracing from systemic intraperitoneal tamoxifen induction demonstrates no evidence of long-term peripheral to central corneal migration of labeled cells. Intraperitoneal tamoxifen was used to induce RFP lineage marker in Myh11+ CECs, with labeling examined 6 and 16 weeks post induction. **(A)** Counts of *Myb11*-Lin(+) RFP-expressing cells in the cornea post-tamoxifen induction show no significant difference. Radial distribution of *Myb11*-Lin(+) cells from periphery (0) to center (1) of the cornea with **(B)** 6 weeks and with **(C)** 16 weeks post induction do not show higher peripheral than central labeling, as would be expected if labeled cells were originating in the periphery and migrating centrally (95% confidence interval of slope in brackets). Representative images from **(D)** 6 weeks and **(E)** 16 weeks post-tamoxifen induction with RFP (red) and DAPI (blue). Scale bar: 1 mm.

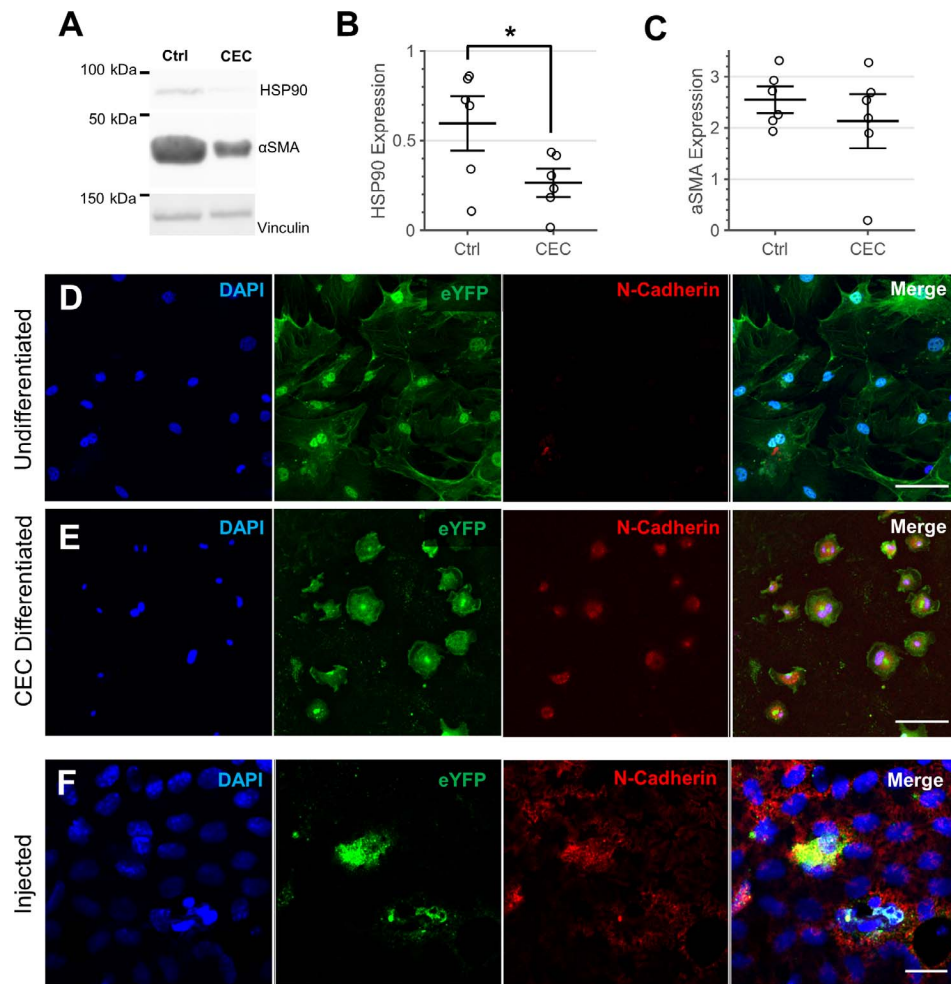
## DISCUSSION

The corneal endothelium is responsible for maintaining optical transparency and nutrient transport for the entire cornea,<sup>30</sup> with CEC loss or dysfunction leading to significant visual impairment that often requires surgical intervention with donor CECs to correct. However, study of individual CEC cell fate has been limited due to the lack of appropriate animal models. We demonstrate for the first time that Myh11 is a marker for a subset of CECs, and show that Myh11 expression can be used in both a temporal and spatial manner to lineage trace CECs to examine their behavior in both healthy and diseased states. Using this model, we show there is no evidence for directional migration of labeled CECs in adult murine corneal tissue. We also show no evidence for acute proliferation of the Myh11 subset of CECs. We further anticipate such a lineage marker could be used to examine the CEC pool to provide additional insight into both embryonic and neonatal development of the cornea. Finally, we suspect that the Myh11 subset of CECs may represent a unique and previously unknown class of CECs that may have functional importance in endothelium maintenance, considering the

established functions of Myh11 in mural cells on the vasculature.

Myh11 has been traditionally thought of as a specific and exclusive marker for vascular smooth muscle cells and pericytes,<sup>14,31</sup> or mural cells that enwrap and regulate the macro- and microvasculature through contraction, juxtacrine, and paracrine signaling. It remains an open question whether there are functional parallels of Myh11 expression in regulating CEC function, given that Myh11 is a major contractile protein in pericytes. Intriguingly, *Myb11*+ CECs lack  $\alpha$ SMA expression, with high  $\alpha$ SMA expression as a defining characteristic of mural cells. Nevertheless, cytoskeletal complexes and other actomyosin proteins are heavily concentrated at the apical tight junctions and adherent junctions that form CEC barrier,<sup>32</sup> and are implicated in the maintenance of CEC barrier integrity.<sup>33-36</sup> Thus, Myh11 may play a key role in regulating CEC permeability through the activation of actomyosin pathways.

Compared with the epithelial and stromal layers, the CEC layer appears to have no regenerative capacity, with substantial evidence pointing to a complete lack of cell turnover, even in the case of acute injury.<sup>37</sup> Accelerated degeneration of the corneal endothelium remains a substantial risk for any of the



**FIGURE 5.** Myh11<sup>+</sup> ASCs can be differentiated into a CEC phenotype and adhere to injured corneal endothelium after delivery into the anterior chamber. ASCs were cultured from epididymal, adipose Myh11<sup>+</sup> mural cells. A lineage marker (YFP) was tamoxifen-induced in these *Myb11*-Lin(+) mural cells before their harvest from adipose tissue. (A–C) Western blot of HSP90 and αSMA expression in *Myb11*-Lin(+) undifferentiated ASCs and CEC-differentiated ASCs, with levels normalized to vinculin. (D, E) Staining with anti-eYFP lineage marker (green), anti-N-cadherin (red), and DAPI (blue) on cultures of (D) undifferentiated and (E) CEC-differentiated *Myb11*-Lin(+) ASCs show upregulation of CEC marker of N-cadherin following 14-day differentiation (scale bar: 10 μm). (F) A scratch wound was made to corneal endothelium with CEC-differentiated *Myb11*-Lin(+) ASCs then injected into the anterior chamber. Seven days post injection, YFP-positive CEC-differentiated *Myb11*-Lin(+) ASCs are found adhering to the corneal endothelial surface with continued expression of N-cadherin. Scale bar: 25 μm.

annual worldwide 185,000 corneal transplants,<sup>38</sup> although cornea transplantation remains the only successful option to partially restore the cornea endothelium. Transplant procedures involve either the replacement of all cornea layers or just the CEC layer and the adjacent basement membrane.<sup>9</sup> These partial transplants use donor CECs in Descemet's membrane endothelial keratoplasty and Descemet's stripping automated endothelial keratoplasty (DSAEK) grafts to replace lost and damaged CECs.<sup>32</sup> However, exogenous cell sources are known to illicit a host immune response, possibly leading to graft rejection, impaired recovery, or a lack of visual acuity improvement.<sup>33</sup> With only 1 in 70 patients in need of corneal transplants actually receiving a graft,<sup>38</sup> there is a chronic shortage of donor tissue to meet global demands.

The use of autologous cell sources for these procedures have the possibility of providing superior clinical outcomes compared with allogeneic transplants,<sup>34</sup> specifically with improved rate of recovery and less likelihood of rejection. Previous work has shown that human umbilical cord-derived stem cells can differentiate into CECs in vitro using differentiation media that alter TGFβ and bone morphogenetic protein

signaling.<sup>26,27</sup> Yet this stem cell source cannot be harvested from adult patients, limiting its utility for large-scale use in the clinic.

ASCs can be harvested in abundance from adult adipose tissue, a readily available adult tissue stem cell source.<sup>35</sup> ASCs also have been shown to have in vitro differentiation capability similar to umbilical cord stem cells. We investigated a specific subpopulation of ASCs, those that express Myh11, and demonstrated they share transcriptional lineage with a subpopulation of CECs, which may possibly confer improved function. The adherence of CEC-differentiated *Myb11*-Lin(+) ASCs to the corneal endothelium surface demonstrates the concept of using an autologous stem cell therapy to regenerate the corneal endothelium, but clearly significant refinement is needed. It may be possible to improve engraftment by preparing a substrate on which CEC-differentiated *Myb11*-Lin(+) ASCs can be grown, allowing for an artificial autologous DSAEK transplant. Future work needs to address maintenance of the CEC phenotype for CEC-differentiated ASCs, improving conditions for engraftment, along with determining the degree



of therapeutic effect of these engineered cells on the recovery of injured corneal endothelium.

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