Existence of Corneal Endothelial Slow-Cycling Cells

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Purpose. To demonstrate the presence and location of corneal endothelial progenitor cells.

Methods. Progenitor cell markers nestin, leucine-rich repeat-containing G-protein–coupled receptor 5, Sox9, and nerve growth factor receptor p75, as well as proliferation marker Ki-67, were examined on postnatal day (P)3, P30, and P90 corneas using immunofluorescence microscopy. Mice (P3) were pulsed with 5-bromo-2′-deoxyuridine (BrdU) and chased.

Results. Cell proliferation was observed in all layers of P3 corneas. No posterior stromal cell proliferation was noted in P30 corneas. Progenitor cell markers were expressed in the P3 cornea, but were downregulated during maturation with minimal or no expression in P90 central corneas. In contrast, cells expressing progenitor markers were located exclusively at the corneal periphery at P90. Clusters of cells reactive for progenitor markers were in the endothelial and subendothelial space in the P90 peripheral cornea. Reactivity against BrdU was localized to the central and peripheral cornea at 1 week, and to the extreme periphery 3 weeks following a BrdU pulse. Cells reactive for both BrdU and progenitor markers were localized to the peripheral endothelium. At 3 weeks, cells reactive for BrdU and the progenitor markers were localized in the peripheral endothelium. Approximately, 20% to 45% of the progenitor marker positive cells also were labeled with BrdU.

Conclusions. During development, the murine corneal endothelium is composed of proliferating cells expressing progenitor markers. In contrast, in the mature endothelium slow-cycling cells, cells expressing progenitor markers and a subpopulation of slow-cycling cells expressing progenitor makers are restricted to the endothelial periphery.

Keywords: corneal endothelium, progenitor cells, slow-cycling cells, nestin, nerve growth factor receptor p75, Sox9

The corneal endothelium is a monolayer of hexagonal cells derived from the neural crest that is responsible for maintaining the appropriate degree of corneal hydration necessary for vision. The normal endothelial cell count is 2 to 5×10^3 cells/mm^2 in humans.[1] An endothelial cell count below a threshold of 300 to 500 cells per mm^2 is associated with irreversible corneal swelling, epithelial blistering, loss of vision, and need of transplantation.[1,2]

The current dogma is that mature corneal endothelial cells are quiescent, arrested in the G1-phase of the cell cycle, with limited proliferative capacity in vivo.[3–5] Cell contact inhibition has been proposed to have a major role inhibiting endothelial replication.[3] However, corneal endothelial cells can replicate in cell cultures and tissue explants. In addition, the presence of endothelial cells expressing progenitor cell markers in the human corneal periphery suggest that endothelial cells or at least some endothelial cells may replicate or have the ability to replicate in an appropriate environment.[6–8] Two different facts strongly suggest that human endothelial cells proliferate in vivo and repopulate donor grafts. First, studies in sex-mismatched transplanted corneas showed the presence of a mixture of donor and recipient endothelial cells in the transplanted graft.[9,10] Endothelial replacement by the host cells in failed edematous corneas, and also in cases where corneas were clear was demonstrated suggesting that endothelial replacement occurs in failed and successful transplants.[9] Second, anecdotal clinical reports show that human endothelial cells can replicate in vivo in the periphery of the posterior cornea and migrate and restore Descemet's membrane, and the endothelial monolayer integrity reestablishing corneal transparency.[11,12]

Nestin, a class VI intermediate filament, has been used to identify cells with progenitor cell properties in the developing neural cortex,[13] cultured primary neurons from the central and peripheral nervous systems,[14] immortalized cell lines, and tumors.[15] It is well documented that expression of nestin reflects the undifferentiated state of neural progenitor cells[16] and that downregulation of nestin reactivity is associated with the differentiation of neural progenitor cells in the developing nervous system.[16] Corneal keratocytes and endothelial cells originate from multipotent cranial neural crest cells that migrate rostrally to form facial structures.[17]

The low-affinity nerve growth factor receptor (NGFR) is one of the two receptor types for the neurotrophins and has been used as a neural crest stem cell marker.[18–20] A pure or enriched population of neural crest stem cells has been isolated from mouse trunk neural tubes and embryonic peripheral nerves using low-affinity NGFR as a marker.[18,19] These cells that are NGFR-positive had self-renewal capacity, and displayed multipotent differentiation properties.

Other progenitor cell markers include Sox9 and leucine-rich repeat containing G protein-coupled receptor 5 (LGR5). Sex-determining region Y-box containing gene 9 (Sox9), high-

mobility group box (Sox) transcription factors, is mutated in campomelic dysplasia, a disorder characterized by skeletal malformations, XY sex reversal, and neonatal lethality.[21] Sex-determining region Y–box 9 is expressed during embryogenesis in several tissues and organs, including the central nervous system.[22] Initially identified as a Wnt target gene in human colon cancer cell lines harboring Wnt-activating mutations,[23] Lgr5 is a well-established epithelial intestinal stem cell marker.[24,25]

Detection of Ki-67 is commonly used in histological specimens to detect cell proliferation and provides an index of proliferation with prognostic value.[26] It was initially reported in the early 1980s.[27] It is a nonhistone nuclear and nucleolar protein strictly associated with cell proliferation and is detected during all the active phases of the cell cycle, but is absent in resting cells. Its strict association with cell proliferation and its coexpression with other well-known markers of proliferation indicate a pivotal role in cell division. Expression of Ki-67 is upregulated in the G_2, M, and latter half of the S phase of the cell cycle.[28]

Progenitor cells are able to differentiate into one or more cell types, but do not have unlimited differentiation or replication properties like stem cells. Progenitor cells can adjust their cell-cycle properties, in homeostasis or during injury, to the circumstances that surround them.[29] A classic way to search for stem cells or progenitors cell niches is the use of labeling agents like 5-bromo-2′-deoxyuridine (BrdU).[29] Slow-dividing cells found in hair follicles and the corneal limbus have been previously described as stem cells.[30] Therefore, this slow-cycling characteristic is considered a common feature to identify stem cells in different tissues and organs.

Here we demonstrate the presence of populations of progenitor cells in the corneal periphery as well as the existence of slow-cycling cells in the extreme periphery of the corneal endothelium.

Materials and Methods

Animals

We used C57BL/6 mice in this study. Corneal tissue from mice at postnatal day (P)3, P30, and P90 was examined. All experiments conformed to the use of laboratory animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care and use committee of the University of South Florida College of Medicine. We used mice at different stages of eye development in this study: P4 (immature corneas); P30 (maturing corneas); and P90 (mature corneas). All experiments were performed at least three times in tissue from at least three different animals.

BrdU Label-Retaining Assay

Mice received intraperitoneal injections of BrdU at 50 mg/kg, (Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in 1X PBS twice daily for 2 days. Mice were then chased for 1 and 3 weeks before being killed. Globes were enucleated and dissected under a microscope. An air bubble was injected into the anterior chamber using a 32-gauge (G) needle to separate the iris from the peripheral cornea before fixation and further processing as flat mounts.

Immunofluorescence Microscopy

Immunofluorescence localization was performed as previously described.[31] Briefly, corneas were fixed with cold methanol or 4% paraformaldehyde in PBS, cryoprotected with sucrose-PBS in a series of dilutions (10%, 20%, and then 30%), embedded and frozen in OCT medium (Sakura Finetek, Torrance, CA, USA). Cross sections of 6 μm were cut using a cryostat (Microm HM 505E; GMI, Ramsey, MN, USA) followed by immunofluorescence localization. For corneal flat mounts, freshly enucleated corneas were removed under an operating microscope (Carl Zeiss Microscopy, Oberkochen, Germany). Cornea flat mounts were digested in 1 mg/mL collagenase solution (Worthington Biochemical Corporation, Lakewood, NJ, USA) in PBS for 7 minutes at 37°C to facilitate antibody penetration into the tissue. No collagenase was used for BrdU or Ki-67 flat mounts which were fixed in cold methanol. For BrdU staining, tissue was treated with 2N HCl at 37°C for 15 minutes to denature DNA and neutralized in boric acid (pH 8.5) 3 times for 5 minutes. Sections and flat mounts were then blocked with 5% bovine serum albumin (BSA) and then incubated overnight at 4°C in anti-nestin at 1:100 (Sigma-Aldrich Corp.), anti-nerve growth factor receptor p75 at 1:100 (Chemicon, Temecula, CA, USA), anti-Sox9 at 1:100 (Chemicon), anti LGR5 at 1:150 (Abcam, Cambridge, MA, USA), anti-Ki-67 at 1:100 (Dako, Denmark) and anti-BrdU (ab-6326, Abcam) followed by Alexa Fluor 565-conjugated goat anti-rabbit IgG or goat anti-rat IgG (Molecular Probes, Eugene, OR, USA) at 1:200. Alexa Fluor 594 phalloidin (Molecular Probes) also was used. Flat mount corneas were divided into 4 symmetrical quadrants. Positive and negative controls were processed in parallel. The nuclei were counterstained using Vectashield mounting solution with DAPI (Vector Lab, Inc., Burlingame, CA, USA). Corneal flat mounts double staining was performed by incubating tissue with primary antibodies overnight. Secondary antibodies were added together and incubated for one hour. Images were captured using a confocal laser-scanning microscope (FV1000 MPE; Olympus America, Inc., Center Valley, PA, USA) with a ×40 or a ×60 1.42 NA oil immersion lens. To avoid bleed-through between fluorescence emissions, samples were scanned sequentially with 488- and 543-nm lasers, and emissions were collected with appropriate spectral slit settings.

Results

An Immature Corneal Endothelial Monolayer Is Mitotically Active

To establish the degree of corneal endothelial maturation during development, immature (P3) and maturing (P30) endothelial monolayers were evaluated. There was a striking difference in the morphology of the endothelial monolayer in flat mounts stained for nuclei (DAPI) and cytoskeletal actin (phalloidin) during endothelial maturation. The immature monolayer consisted of a disorganized array of cells. The cells had irregular shapes, large nuclei/cytoplasm ratio, thin and irregular cell outlines, and occasional cell clusters were noted. Only one or two clusters were noted in a corneal quadrant (Fig. 1A). In contrast, in the maturing cornea more hexagonal, thicker cell membrane, smaller nuclei/ratio, and well organized endothelial cells, and no cell clusters were observed (Fig. 1B).

Endothelial cell proliferation was analyzed in both P3 and P30 corneas using the proliferation marker Ki-67. Expression of Ki-67, a nuclear protein associated with cell proliferation, was noted in all layers of the P3 cornea. No significant differences in Ki-67 expression between the central cornea or corneal periphery or between the anterior or posterior cornea were observed (Figs. 2A, 2B, central and limbal cornea, respectively). In the P30 cornea, Ki-67 expression was only observed in the basal layers of the epithelial layer (Fig. 2C). Expression of Ki-67 decreased with maturation of the endothelial monolayer as noted in endothelial flat mounts (Fig. 3). A labeling index of Ki-67, percentage of positive cells

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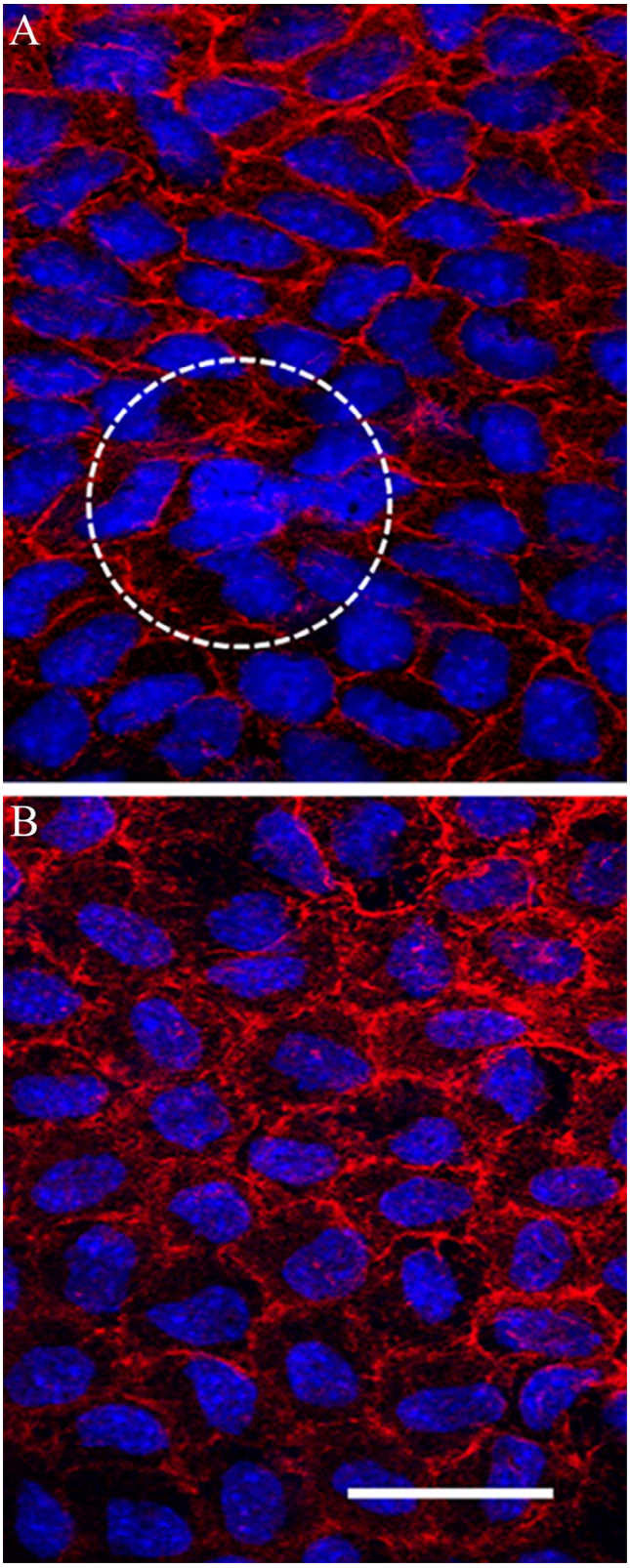

Figure 1. Endothelial maturation in the P3 and P30 cornea. The immature monolayer consists of irregularly shaped cells with large nuclei (*blue*) and a thin cytoplasm. Endothelial cell outlines demonstrated by cortical actin staining (*red*) is poorly organized and thin. Occasional cell clusters are seen in the monolayer, white circle (**A**). In contrast, the P30 monolayer consists of regularly shaped hexagonal cells, a smaller nuclei/cytoplasm ratio, and more round nuclei (**B**). Fluorescence microscopy of endothelial flat mounts reacted with Phalloidin (*red*) for F-actin and DAPI (*blue*) for nuclei. *Scale bar*: 50 μm.

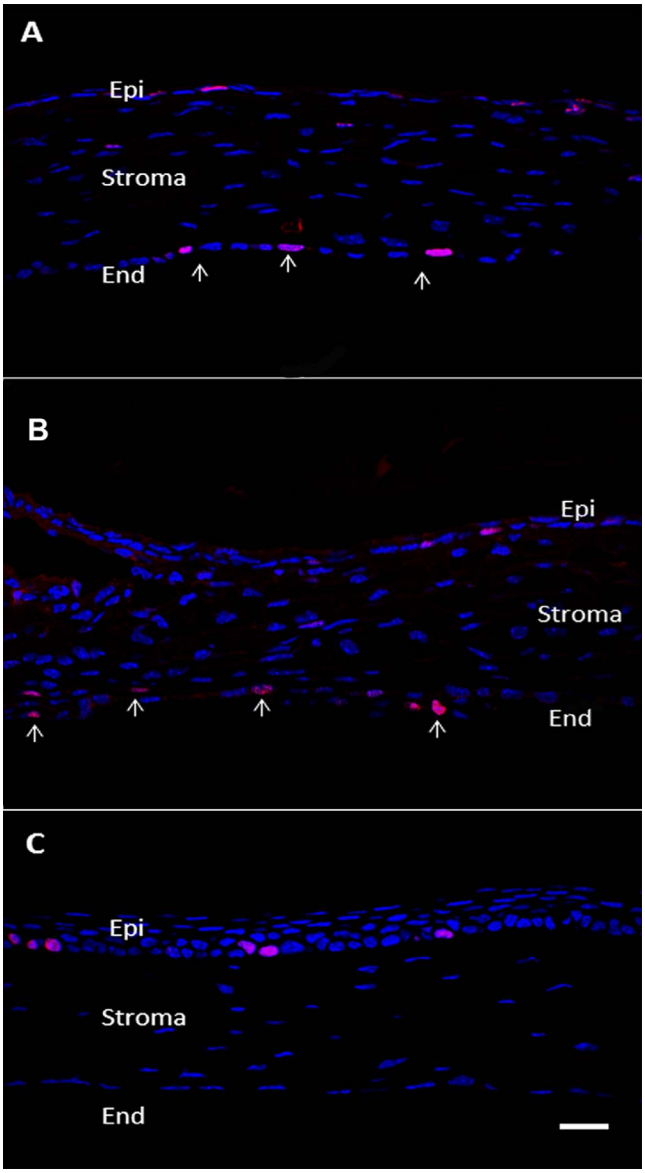

Figure 2. Proliferative Capacity in the P3 and P30 cornea. No difference in proliferation was noted between the central or peripheral cornea. Ki-67 reactivity (red nuclei), was noted in all layers of the central (**A**) and limbal P3 cornea including the endothelial monolayer (**B**). In contrast, Ki-67 staining, red nuclei, is only expressed in the epithelium of the P30 cornea (**C**). Immunofluorescence microscopy of corneal cross-sections reacted with anti-Ki67 (*red*) and DAPI (*blue*) for nuclei. *Scale bar*: 25 μm.

in corneal flat mounts, decreased from the central cornea to the periphery in the P3 mouse. The labeling index of Ki-67 was 7.9 ± 0.6 in the central cornea compared with 3.9 ± 0.4 in the peripheral cornea. No expression of Ki-67 was noted at age P30 neither in the central or peripheral cornea. These data indicate that stromal and endothelial cells are actively undergoing replication in the P3 cornea and are quiescent in the P30 cornea.

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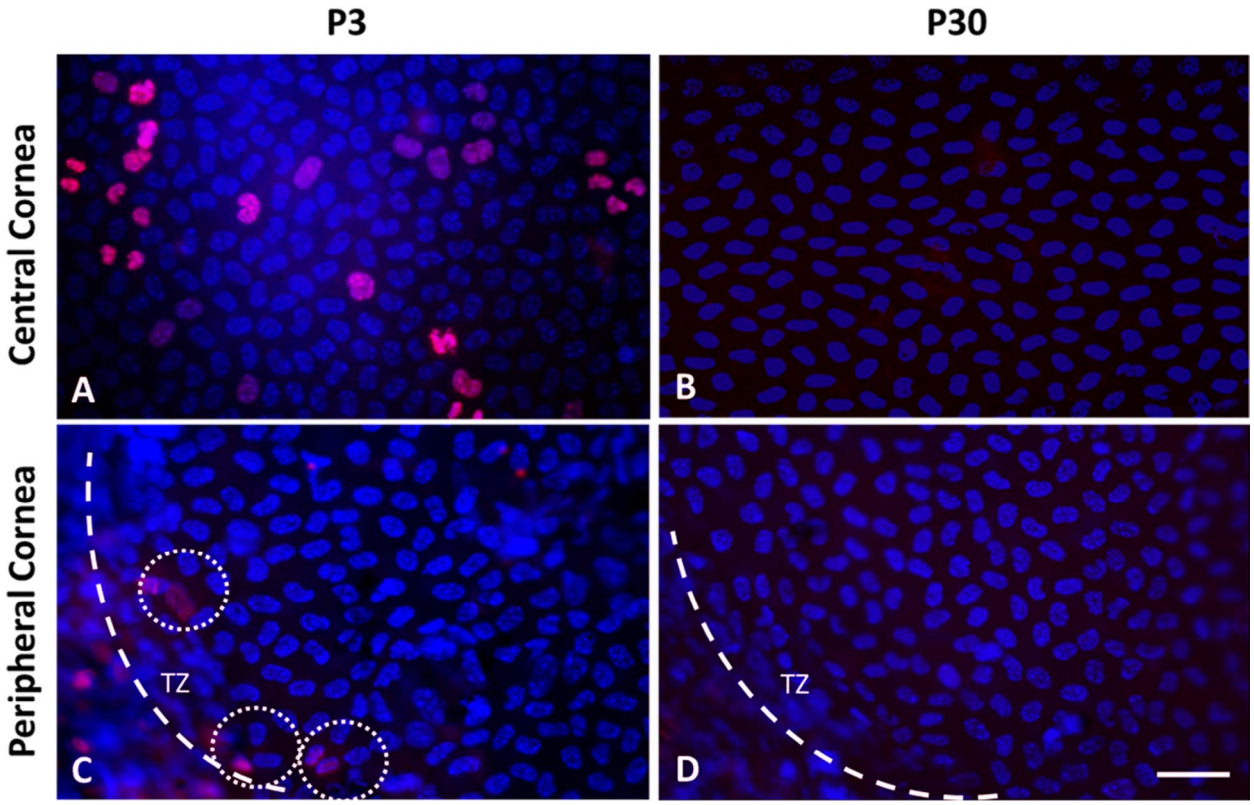

Figure 3. Endothelial cell proliferation in the P3 and P30 cornea. Endothelial proliferation was analyzed in flat mounts using an antibody directed against Ki-67 and immunofluorescence microscopy. A significant percentage of endothelial cells are undergoing cell replication in the central cornea of P3 mice (**A**). No mitosis is noted in the central cornea of the P30 mice (**B**). A few cells are reactive against Ki-67 in the peripheral cornea of P3 mice (**C**), but in the peripheral P30 cornea, no Ki-67 reactive cells are observed consistent with mitotically quiescent cells (**D**). Immunofluorescence microscopy of the corneal endothelium in flat mount preparations reacted with anti-Ki67 (*red*) and DAPI (*blue*) for nuclei. *Scale bar*: 40 μm.

Nestin Expression Localizes to the Cornea Periphery With Maturation

In the immature cornea (P3), nestin is expressed by epithelia, keratocytes, and endothelia. All cell populations demonstrate strong nestin reactivity in both the central (Fig. 4A, inset shows endothelial cells at higher magnification) and peripheral cornea (Fig. 4B, inset shows endothelial cells at higher magnification). In the P30 cornea, central corneal epithelial cells express nestin predominantly in the basal epithelial layer. There is weak nestin reactivity in keratocytes and endothelial cells (Fig. 4C). However, in the peripheral cornea, nestin expression is maintained throughout the full thickness of the epithelial layer, minimal staining is noted in the stromal keratocytes while endothelial cells still express nestin (Fig. 4D, small arrows). In the P90 cornea, very weak or no reactivity was noted in the central cornea in epithelium, keratocytes, and endothelium (Fig. 4E). In contrast, nestin expression localizes to the corneal periphery endothelium and trabecular meshwork region (Fig. 4F, small arrows). In summary, expression of progenitor marker nestin, present in the entire immature endothelial monolayer becomes localized only to the periphery in the P30 and P90 corneas. This finding suggests a changing distribution of endothelial progenitor cells with maturation where localization in the mature cornea is exclusively at the endothelial monolayer periphery.

NGFR Is Differentially Localized to the Peripheral Cornea During Maturation

In the P3 cornea, NGFR is expressed by epithelial cells, keratocytes and endothelium of the central cornea (Fig. 5A) as well as peripheral cornea (Fig. 5B). The expression pattern of NGFR is similar to that seen for nestin in the immature cornea. In postnatal day 30 cornea, NGFR expression is maintained in the full thickness of the epithelial layer and minimal staining is noted in the keratocytes of the central cornea and corneal periphery. Endothelial NGFR expression is stronger in the corneal periphery compared with the central cornea (Figs. 5C, 5D, respectively, asterisks show immunoreactivity). In the postnatal day 90 cornea, NGFR expression had a similar pattern to nestin expression. Immunoreactivity was very weak or no reaction was noted in the epithelial cells, keratocytes, or endothelium of the central cornea (Fig. 5F, shown by small arrows). In contrast, reactivity was noted in the endothelium and trabecular meshwork region of the corneal periphery (Fig. 5E). Therefore, analogous to nestin, the expression of NGFR also becomes localized to the periphery in the mature endothelial monolayer.

Sox9 and LGR5 Are Downregulated With Endothelial Maturation

In the P3 cornea, LGR5 is diffusely expressed by epithelial cells, keratocytes, and endothelium of the central cornea (Fig. 6A). The pattern of SOX9 expression is very similar with a predominant nuclear staining by epithelial cells, keratocytes, and endothelium in the P3 central cornea (Fig. 6B). In postnatal day 30 corneas, LGR5 expression was not observed in the central cornea (Fig. 6C); similarly, no SOX9 expression was noted (Fig. 6D). Endothelial LGR5 and SOX9 expression

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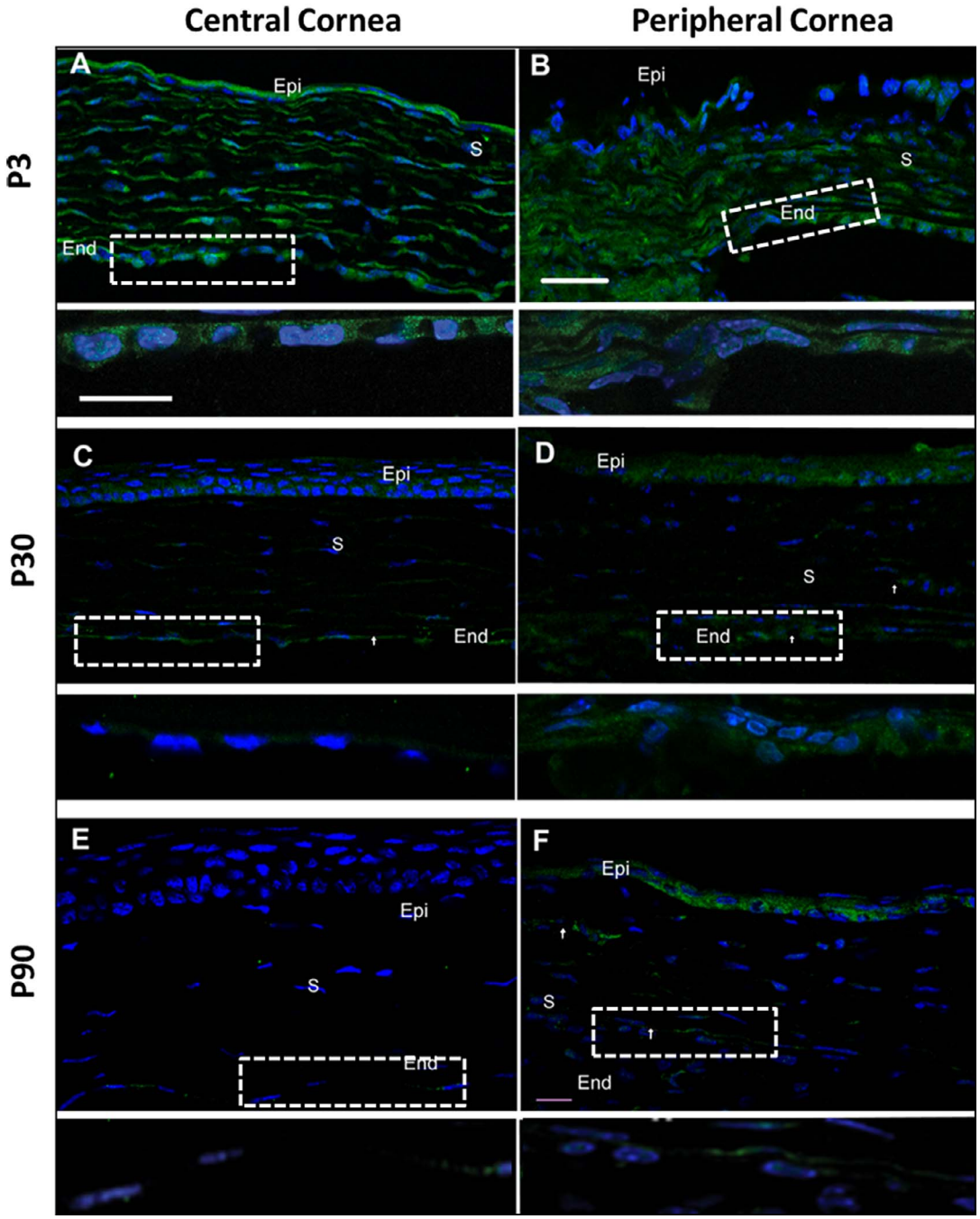

Figure 4. Nestin expression localizes to the corneal periphery during corneal maturation. The epithelial, stromal, and endothelial cells in the central (**A**) and peripheral cornea (**B**) are reactive against nestin at P3. A similar pattern is noted at P30, but nestin expression is downregulated in the corneal periphery and central cornea (**C**, **D**). At age P90, no staining is noted in the central cornea (**E**). Nestin is expressed only in the basal epithelium and endothelium of the corneal periphery (**F**). Insets show endothelial cells at higher magnification. Immunofluorescence microscopy of corneal cross-sections reacted with anti-nestin (*green*) and DAPI (*blue*) for nuclei. *Scale bar*: 25 μm. *Inset bar*: 20 μm.

was stronger in the corneal periphery compared with the central cornea (Figs. 6E and 6F), respectively.

Nestin and NGFR-Positive Cell Clusters Reside at the Periphery of the Adult Cornea

To better evaluate the expression of progenitor markers in three dimensions and presence of progenitor cells in the posterior stroma, collagenase treated flat mounts were examined with the endothelium side up. These preparations demonstrated NGFR reactivity in endothelial cells located in the corneal periphery at the transition zone, between cornea and trabecular meshwork. Nestin reactive cell clusters were also localized in the subendothelial space of both the corneal midperiphery (Fig. 7A) and close to the transition zone next to the trabecular meshwork (Fig. 7B). Reactivity for NGFR also showed positive clusters of subendothelial cells at the transition zone next to the trabecular meshwork (Fig. 7C).

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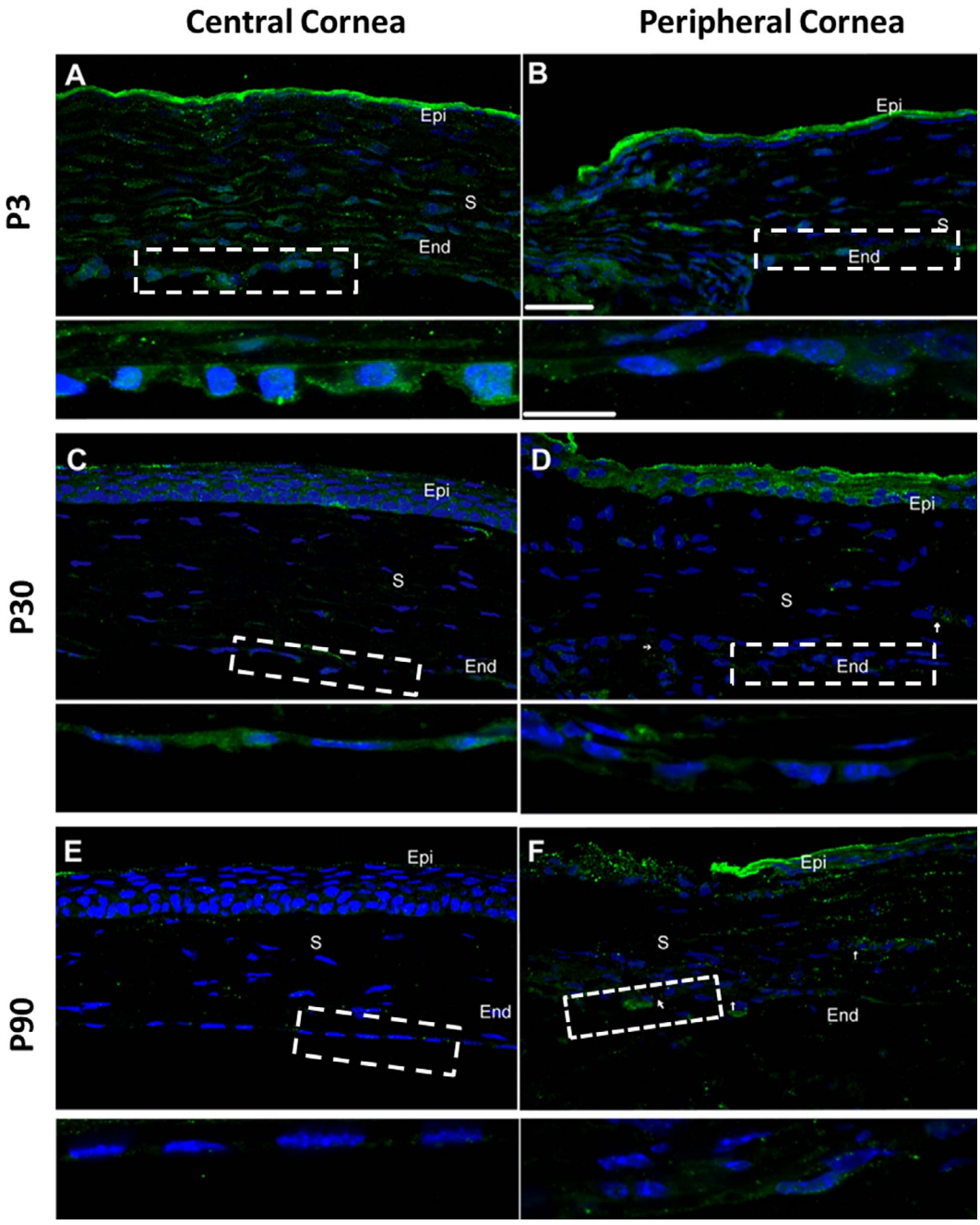

Figure 5. Expression of NGFR is maintained in the corneal periphery during corneal maturation. At P3, the epithelial, stromal, and endothelial cells in the central cornea (**A**) and corneal periphery (**B**) are reactive against NGFR. At P30, NGFR expression is downregulated in the corneal periphery and central cornea, and only epithelial cells show strong reactivity (**C**, **D**). At P90, NGFR is strongly expressed in the epithelium and no staining is noted in the central cornea stroma or endothelium (**E**). Some reactivity is seen in the stromal, shown by *asterisks*, and endothelium of the corneal periphery (**F**). *Insets* show endothelial cells at higher magnification. Immunofluorescence microscopy of corneal cross-sections reacted with anti-NGFR (*green*) and DAPI (*blue*) for nuclei. *Scale bar*: 25 μm. *Inset bar*: 20 μm.

Slow-Cycling, Label-Retaining Cells Reside in the Extreme Periphery of the Corneal Endothelium

Slow-cycling, label-retaining cells are an essential feature of stem cell niches. In the eye, the sclerocorneal limbus is a niche that harbors slow-cycling cells with a high capacity for regeneration of corneal epithelium.[30] Our data demonstrate that progenitor cells reside in the periphery of the endothelial surface. To demonstrate that slow-cycling, label-retaining cells are present in the same peripheral endothelial surface where progenitor cell reside, BrdU labeling was performed in flat mounts to evaluate the endothelial surface, 1 and 3 weeks post-BrdU pulse injections. Scattered BrdU expression was observed in the central and peripheral endothelial surface at 1 week postinjection; 9.6% ± 2.7% of cells expressed BrdU at 1 week postinjection (Figs. 8A–C). In contrast, 3 weeks post–pulse injections, BrdU reactivity was completely absent in the central cornea (Figs. 8D, 8E), and was present exclusively in the extreme endothelial periphery (Fig. 8F), in proximity to the trabecular meshwork and iris. Only 0.8% ± 0.2% of endothelial cells expressed BrdU 3 weeks post–pulse injections.

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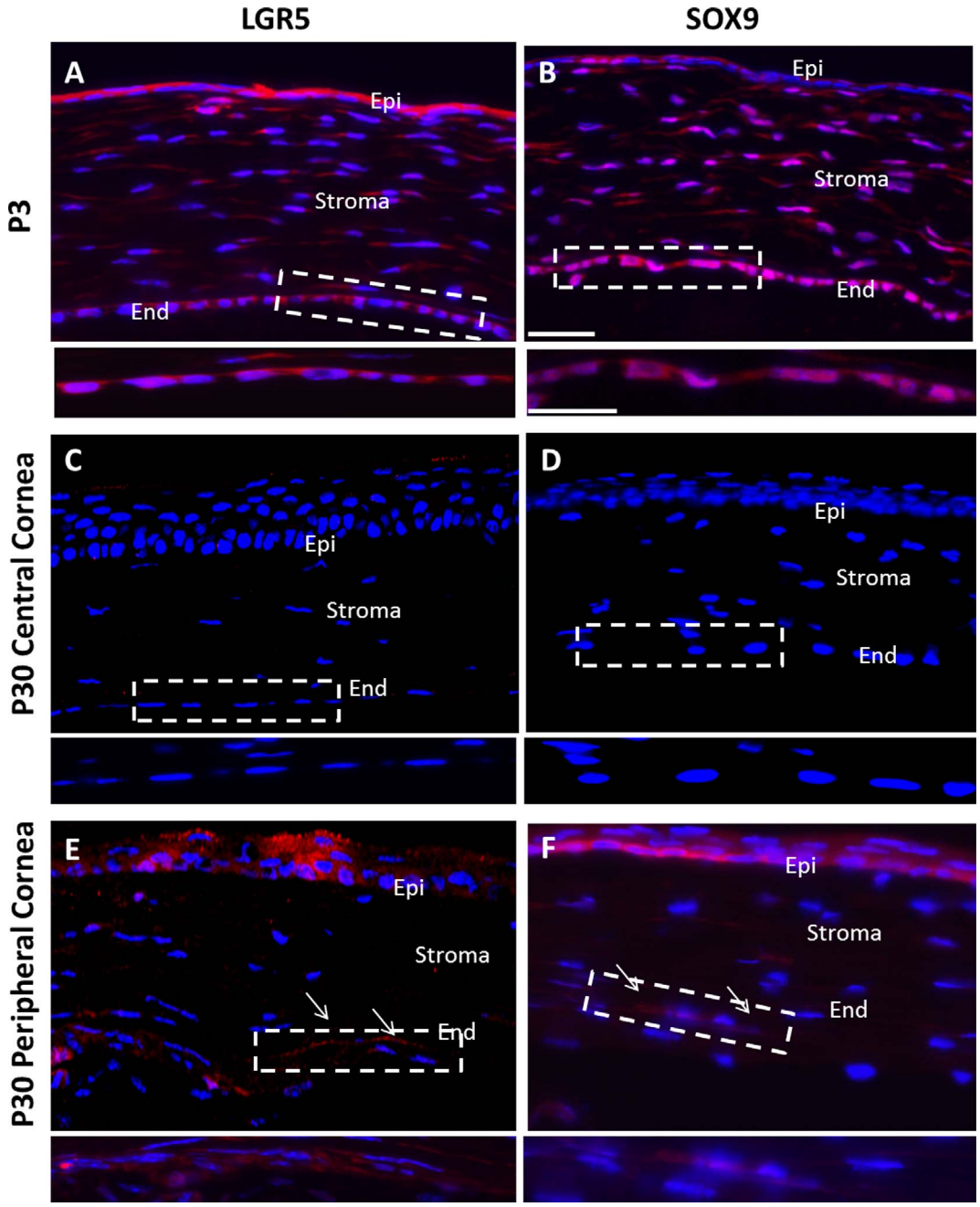

Figure 6. Expression of the progenitor cell markers LGR5 and Sox9 is downregulated with corneal maturation. Expression of LGR5 and Sox9 are noted in the epithelial, stromal, and endothelial cells of the immature eye P3 (**A**, **B**). In the P30 cornea, LGR5 and Sox9 are not expressed by keratocytes or endothelial cells (**C**, **D**). Cell markers LGR5 and Sox9 are only expressed by some endothelial cells: keratocytes and epithelial cells located in the peripheral cornea (**E**, **F**). Immunofluorescence microscopy of corneal cross-sections reacted with anti-LGR5 or Sox9 (*red*) and DAPI (*blue*) for nuclei. *Scale bar*: 25 μm, *Inset bar*: 20 μm.

Slow-Cycling Cells Express Progenitor Markers

We investigated whether slow-cycling peripheral endothelial cells also expressed progenitor cell markers. Double staining against progenitor markers and BrdU in corneal flat mounts demonstrated the presence of peripheral endothelial cells with reactivity against both BrdU as well as NGFR (Fig. 9A), LGR5 (Fig. 9B), and Nestin (Fig. 9C). A substantial percentage of BrdU-positive cells also were positive for progenitor markers. Specifically, 46.8%, 21.7%, and 23.1% coexpressed nestin, LGR5, and NGFR, respectively. A comparable range of the cells positive for the progenitor markers, nestin, LGR5, and NGFR were positive for BrdU, 21.1%, 40.0%, and 35.7%, respectively. These data support a subpopulation of slow-cycling cells that express progenitor markers.

We can draw two conclusions from these findings: endothelial cells replicate actively during early postnatal life; and the mature endothelial surface harbors slow-cycling, label-retaining cells, expressing progenitor markers that reside in the extreme periphery.

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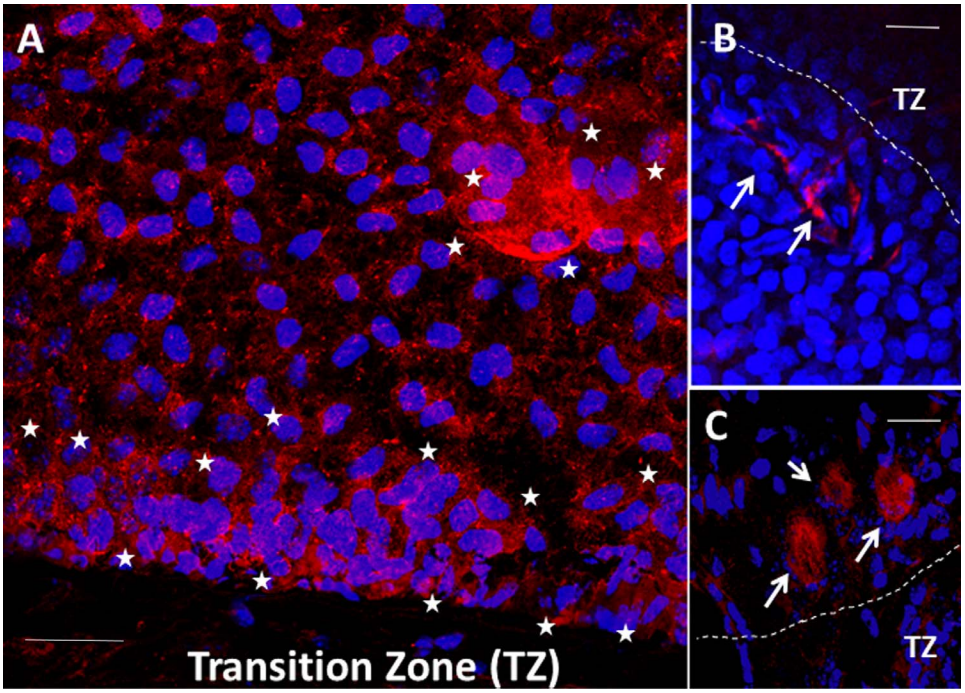

Figure 7. Cells expressing nestin and NGFR reside in the periphery of the maturing endothelium. Flat mount immunolocalization of NGFR showed positive cells at the junction of the corneal endothelium monolayer and the trabecular meshwork, shown by *stars*, as well as cell clusters under the endothelium, shown by *stars* (**A**). Clusters of positive NGFR cells also were seen in the subendothelial stroma of the corneal periphery close to the transition zone to the trabecular meshwork, shown by *arrows* (**B**). Subendothelial nestin–positive cell clusters were noted in the corneal periphery, marked by *arrows*. (**C**) Immunofluorescence microscopy of corneal flat mounts reacted with anti-nestin orNGFR (*red*) and DAPI (*blue*) for nuclei. *Scale bar*: 30 μm.

Discussion

Loss of endothelial function is a major indication for corneal transplantation. Progress in the understanding of corneal endothelial biology, the presence and location of progenitor cells and whether this is a population that can be recruited to aid in restoration of a functional endothelial monolayer is essential to advance new surgical techniques and develop endothelial regeneration. Herein we demonstrate that slow-cycling cells and cells expressing progenitor markers are restricted to the extreme periphery of the mature corneal endothelium. The location of slow-cycling, label-retaining cells in the extreme periphery is suggestive of the existence of a peripheral endothelial niche.

This and our previous work suggest that endothelial maturation and differentiation is a process regulated by the surrounding environment that involves anatomical, functional and proliferative changes.[31] During endothelial maturation, cells differentiate and acquire a mature phenotype, able to maintain appropriate corneal hydration. A striking finding in the immature mouse corneal endothelium is the presence of intracellular and subbasal vesicles that are not present in the mature cornea. Also, diffuse pattern of ZO-1 staining in the P14 mice became more organized and localized to the basolateral cell membranes of maturing corneas.[31]

We believe that the regenerative capacity of endothelial cells evolves along with the anatomical and functional properties of the maturing endothelium. Our findings demonstrate that immature endothelial cells in the entire endothelial sheet have phenotypic characteristics of progenitor cells, with positive staining for different progenitor markers including nestin, NGFR, Sox-9, and LGR5. However, during normal cornea maturation, immature endothelial cells differentiate to functional adult cells that lose their replicative properties and become quiescent. By analyzing Ki-67 proliferation marker expression and labeling cells with BrdU at different ages, we found that proliferation in the unwounded cornea is active in early postbirth days, but ceases around days P10 to P12. Therefore, a major change of endothelial maturation includes endothelial cells losing the ability to reproduce. A young immature endothelium actively replicates and is composed of progenitor cells in contrast to a mature functional endothelial monolayer.

In this work, we demonstrated the presence of strong peripheral endothelial expression of four progenitor cells markers in the mature murine cornea. Based on analyses of the controls and comparable results with four different markers, the data support the presence of a progenitor population in the mature peripheral mature corneal endothelium. In addition, we demonstrate a subpopulation of progenitor cells that are sequestered in the peripheral mature cornea and have slow-cycling properties. We demonstrate that ~20% to 45% of these cells also express progenitor markers. We hypothesize that this undifferentiated endothelial progenitors cells that retained BrdU and are present in the extreme periphery of the mature corneal endothelium are cells that may be recruited for tissue repair and regeneration. Based on the data, we suggest the localization of these cells to the extreme corneal periphery points to the presence of a corneal endothelial niche. Similar findings are reported in human corneas where a unique progenitor cell population and anatomy exist in the corneal periphery.[6–8,32]

The subpopulation of peripheral slow-cycling cells that coexpress progenitor markers may be underrepresented in this work. A discrete population of slow-cycling cells was defined in the periphery of the mature endothelium. However, the number may be underrepresented in our study. Injecting adult mice with 5-bromo-2′-deoxyuridine during 48 hours might fail

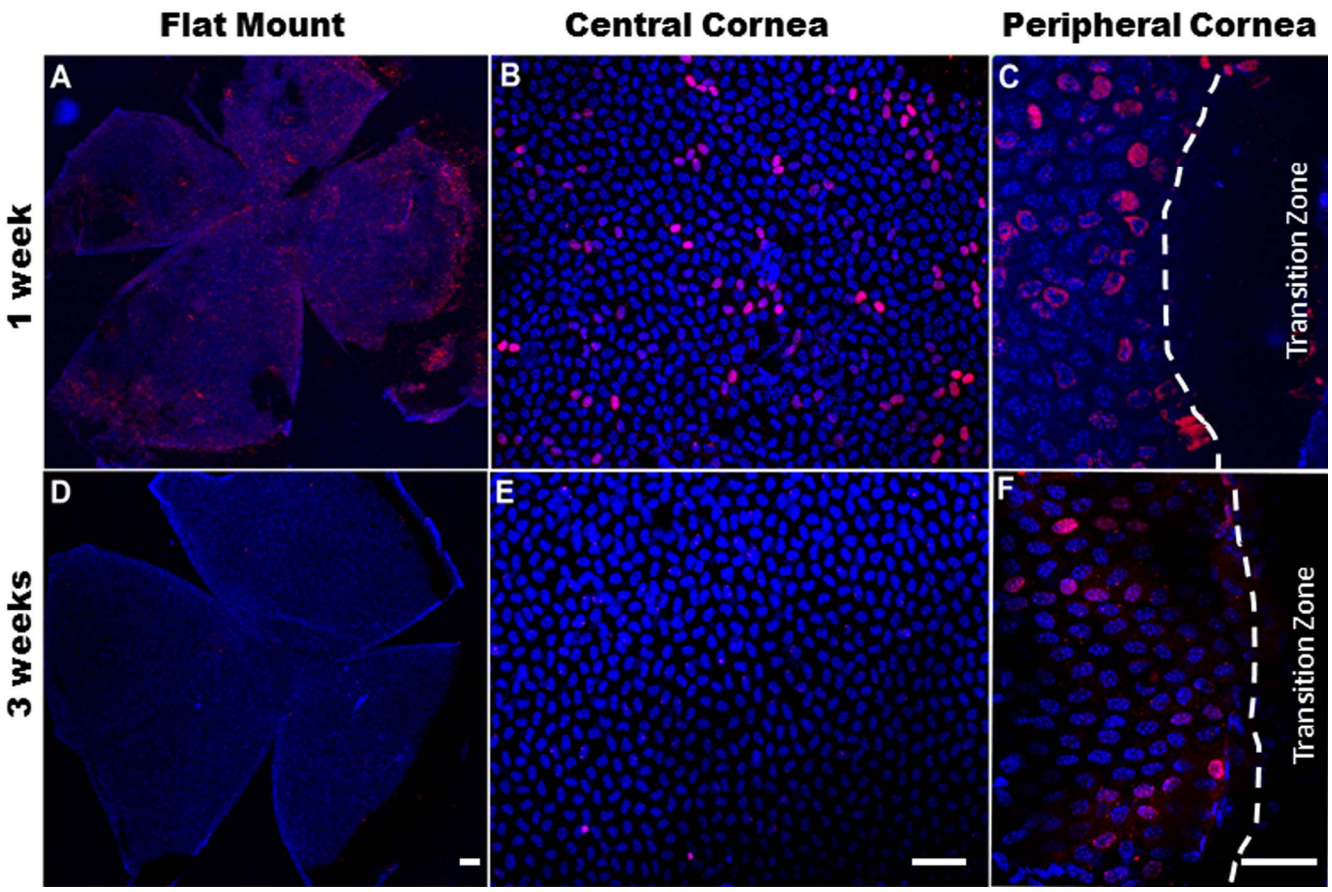

Figure 8. Slow-cycling cells localize to the extreme periphery of the maturing corneal endothelium. Mice were injected with BrdU, chased for 3 weeks, and analyzed at P30 using fluorescence microscopy. Reactivity against BrdU was scattered in the entire corneal endothelial surface following a 1-week chase (**A**). The cornea showed clusters of replicating cells in the central (**B**) and peripheral cornea (**C**). No apparent BrdU staining is noted after a 3-week chase at low magnification (**D**). We noted no BrdU reactivity in the central cornea (**E**). Slow-cycling, BrdU-positive cells are exclusively localized to the extreme corneal periphery (**F**). Immunofluorescence microscopy of corneal flat mounts reacted with anti-BrdU (*red*) and DAPI (*blue*) for nuclei. *Scale bar*: 50 μm.

to label highly quiescent stem cells that may not divide frequently enough; and while this work demonstrates and localizes a distinct subpopulation of slow-cycling cells; future work will be required to refine the numbers. We found that ~20% to 45% of these cells were reactive for BrdU and coexpressed progenitor markers depending on how the analysis was done. We believe that this percentage is probably an underestimate of the true double-labeling percentages. First, BrdU pulse protocol may not optimally label all slow-cycling cells. This can be addressed in future work with earlier ages during development and for longer pulses to label all slow-cycling cells. Second, although we used different progenitor cells markers, no single stem cell marker has been clearly established as an endothelial progenitor stem cell marker and the endothelial progenitor phenotype is unlikely to be defined by a single marker, but rather by a panel with differing expression at different stages. Finally, there are technical issues involving acid pretreatment necessary for BrdU and antibody penetration common to these approaches that would reduce the number of cells with both attributes that were labelled in these experiments.

It is unknown what mediates the inhibition of proliferation in mature corneal endothelial cells and what mechanism could activate endothelial cells to replicate in cases of injury. Some authors believe that the formation of endothelial cell contacts plays a role in inhibition of proliferation in the mature endothelium. Joyce et al.[3] suggest that the formation of cell contacts is associated with an increase in the expression of p27Kip1 protein. However, new surgical techniques suggest that activating mature endothelial cell replication is feasible. Lam et al. are currently improving a new successful surgical procedure for endothelial repair, called Descemet's membrane transfer, where a piece of Descemet's membrane is inserted into the anterior chamber and allowed to float.[33,34] It is therefore critical to identify the regulation of corneal progenitor cells and the influence of the surrounding environment as a first step for endothelial bioengineering and surgical replacement therapies. Other groups are able to isolate and expand progenitor cells obtained from human and mice and demonstrate with transplantation their functionality.[32,35]

It is interesting to note the presence of strong peripheral epithelial expression of all four progenitor cells markers studied here. Based on comparisons with controls and comparable results with four different markers, we believe that this is specific.

We believe that there are multiple clinical implications to the findings described. The preservation of an intact peripheral "endothelial niche" in cases of endothelial failure localized to the central cornea like in Fuchs endothelial dystrophy could explain the better long-term graft survival noted in Fuchs endothelial dystrophy compared with advanced aphakic or pseudophakic bullous keratopathy.[36] If the entire corneal endothelium monolayer, including the "endothelial niche" is

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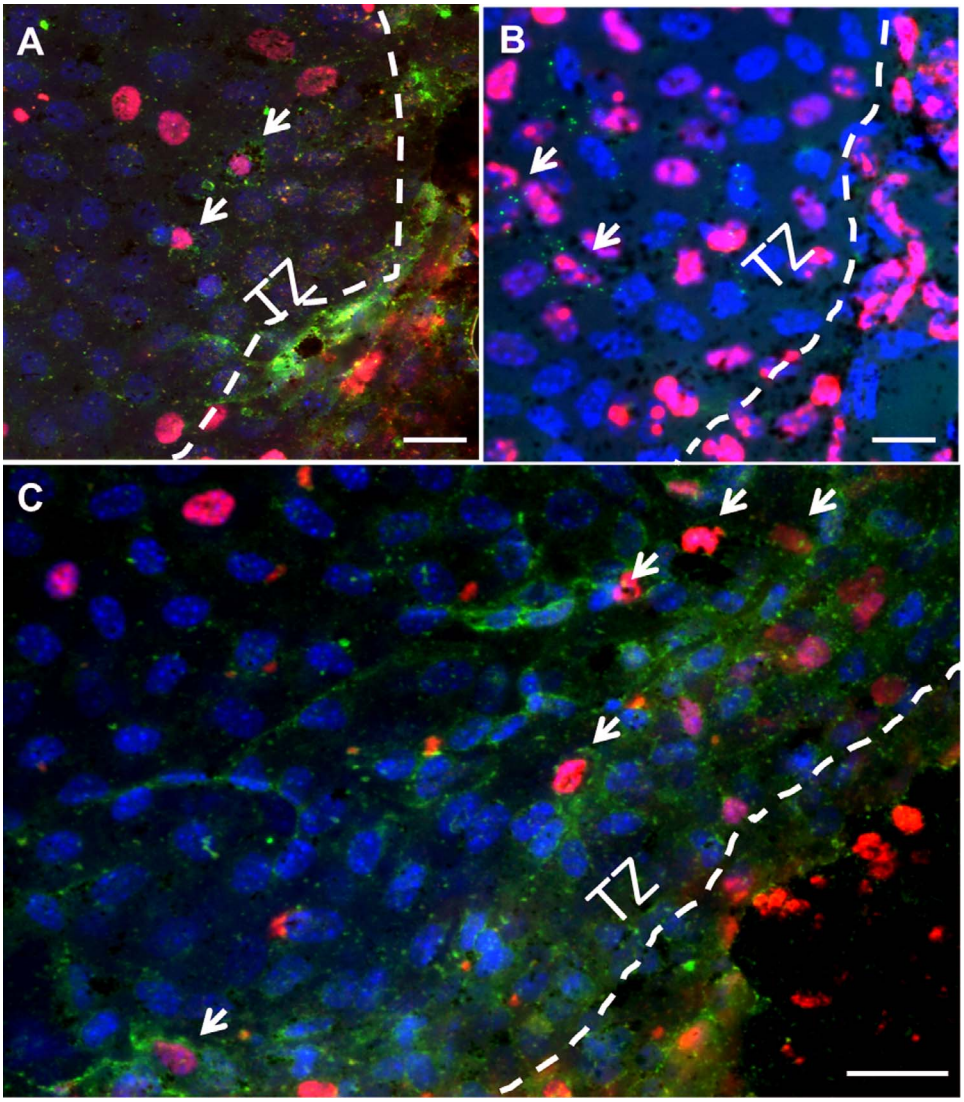

Figure 9. Colocalization of BrdU and proliferation markers in the periphery of the mature corneal endothelium. Representative micrographs demonstrating that a subset of BrdU-positive cells coexpress progenitor cell markers in the extreme periphery of the corneal endothelium. Approximately 20% to 45% of BrdU-positive cells also express progenitor markers. Expression of NGFR was noted in few BrdU-retaining endothelial cells, noted by *arrowheads* (**A**). Coexpression of LGR5 was also noted in some BrdU-retaining cells, noted by *arrowheads* (**B**). Nestin was expressed in the endothelial periphery by some BrdU-retaining cells, noted by *arrowheads* (**C**). Fluorescence microscopy of corneal flat mounts after reactivity for BrdU (*red*) and nestin, NGFR, or LGR5 (*green*) and DAPI (*blue*) for nuclei. *Scale bar*: 40 μm.

compromised, graft survival would be also impaired. If there is an endothelial peripheral niche responsible to sustain endothelial cells viable in the donor graft, should surgery be performed earlier to protect the "endothelial niche" before stromal edema compromises the niche? Other serious conditions affecting corneal transparency and that may be secondary to dysregulation of the endothelial niche and its progenitor cells are the iridocorneal syndrome where epithelial like cells from the periphery invade the corneal endothelium.

In conclusion, we demonstrate that the process of corneal endothelial maturation includes restriction of progenitor cells to the corneal periphery. These findings point to the presence of an endothelial niche in the corneal periphery of the adult corneal endothelium. Defining the mechanisms that regulate the structure and function of the endothelial niche would open multiple translational opportunities to prevent corneal diseases and to improve corneal transplant survival and transparency.

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