



Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas

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Purpose: The corneal endothelium is a monolayer of cells in the posterior cornea that is responsible for maintaining a clear cornea. Corneal endothelial cells may be induced to divide, but it has been held that they do not divide in the normal cornea of an adult human. Some studies have suggested that a stem cell population for the corneal endothelium exists. This population could give rise to mature corneal endothelial cells and may reside either in the peripheral corneal endothelium or in the adjacent posterior limbus. This study was initiated to demonstrate the presence of such stem cells in the region of the posterior limbus and to show the response of these cells to corneal wounding.

Methods: Unwounded and wounded corneas with their attached limbal sections were analyzed by immunofluorescence for the presence of nestin, telomerase, Oct-3/4, Pax-6, Wnt-1, and Sox-2. Alkaline phosphatase activity was observed with an enzyme-based reaction that produced a fluorescent product.

Results: In the unwounded cornea, stem cell markers nestin, alkaline phosphatase, and telomerase were found in the trabecular meshwork (TM) and in the transition zone between the TM and the corneal endothelial periphery (including Schwalbe's line). Telomerase was also present in the peripheral corneal endothelium. When wounded corneas and their attached limbi were tested, the same markers were found. However, after wounding, additional stem cell markers, Oct-3/4 (in the TM) and Wnt-1 (in both the TM and the transition zone), appeared. Moreover, the differentiation markers Pax-6 and Sox-2 were seen. Pax-6 and Sox-2 were also manifest in the peripheral endothelium post-wounding.

Conclusions: Well-documented specific stem cell markers were found in the TM and the transition zone of the human posterior limbus. Wounding of the corneas activated the production of two additional stem cell markers (Oct-3/4, Wnt-1) as well as two differentiation markers (Pax-6, Sox-2), the latter of which also appeared in the corneal endothelial periphery. It is suggested that stem cells reside in the posterior limbus and respond to corneal wounding to initiate an endothelial repair process. The stem cells may also contribute to a normal, slow replacement of corneal endothelial cells.

The corneal endothelium is a monolayer of cells at the posterior of the cornea. These cells are important for maintaining deturgescence, the clear state of the cornea. The endothelial cells accomplish this by transporting fluid out of the stroma and into the aqueous. Although corneal endothelial cells may be induced to undergo cell division, they do not seem to divide in a human beyond the age of 20 [1-8]. However, recent evidence has suggested that there may be a population of precursor cells in the peripheral endothelium or in the posterior limbus (Figure 1 and Figure 2) that can give rise to differentiated endothelial cells [9-12]. To determine the existence of a stem cell population in the posterior limbus, this study investigated the presence of specific molecular markers of stem cells in the corneal endothelium and the posterior limbus.

Evidence suggesting the existence of a stem cell population for the replacement of corneal endothelial cells has come from several sources. Schimmelpfennig [9] and Daus et al. [10] showed an increase in cell density at the peripheral corneal endothelium. Amann et al. [11] reported a 10% average

increase in cell density in the peripheral regions of the human corneal endothelium and proposed a possible "regenerative zone". In 2005, Whikehart et al. [12] demonstrated that telomerase, a marker of transient amplifying cells and stem cells, is expressed in the peripheral endothelium and that bromodeoxyuridine (BrdU) labeling, a marker of cell division, occurs in the trabecular meshwork and posterior limbus. This division was amplified and extended into the endothelium in response to wounding.

This study examined the possibility that stem cell populations exist in the posterior limbus of unwounded and wounded corneas by using the following histochemical markers: the stem cell proteins nestin, alkaline phosphatase, telomerase, Wnt-1, and Oct-3/4 as well as the differentiation markers Pax-6 and Sox-2. Identifying a stem cell population in the posterior limbus can provide insight about the source of corneal endothelial cells. Moreover, the eventual isolation of endothelial precursor cells would be useful for corneal endothelial regeneration as well as the growth of corneal endothelial cells in culture.

METHODS

Processing of corneal tissue: Unwounded human donor corneas were acquired from the Alabama Eye Bank and the Geor-

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gia Eye Bank. Wounded corneas, the outer tissues remaining from corneal transplants, were donated by Dr. Roswell Pfister. Such tissues were considered wounded due to the removal of the central corneal region for transplant by trephination. Fifty-six donor corneas were used in this study, ranging in age from 16 to 73 years old with a mean age of 45 ± 18 years (standard error of the mean). All tissues were kept in a corneal storage media of either Optisol (Chiron Ophthalmics, Irvine, CA) or Eusol-C (Alchimia, Padua, Italy) before fixation. Unwounded tissues were fixed in ethanol (70% pure ethanol containing 50 mM glycine at pH 2.0) for 2-3 h at -20°C within 24 h of receipt. Wounded corneas acquired within 24 h after surgery were placed at 4°C for 24-48 h before fixation to allow for a wounding response [13]. All corneas were stored in phosphate

buffered saline (PBS; 11.9 mM sodium and potassium phosphate, 137 mM sodium chloride, and 2.7 mM potassium chloride at pH 7.4) after fixation. The unwounded and wounded corneas used in this study are considered to be in a "non humans" exempt category in which they have been removed from cadavers before use. The techniques for their use have been reviewed and approved by the IRB for Human Use Committee of the University of Alabama at Birmingham.

Preparation of transverse sections: Fixed corneas were immersed in increasingly higher concentrations of sucrose in deionized water (10%-30%) for 15 min in each solution. Sucrose was added to protect the tissue during freezing and sectioning. The sections were cut in half and frozen in a sectioning mold in a 2:1 solution of 30% sucrose and HistoPrep Fro-

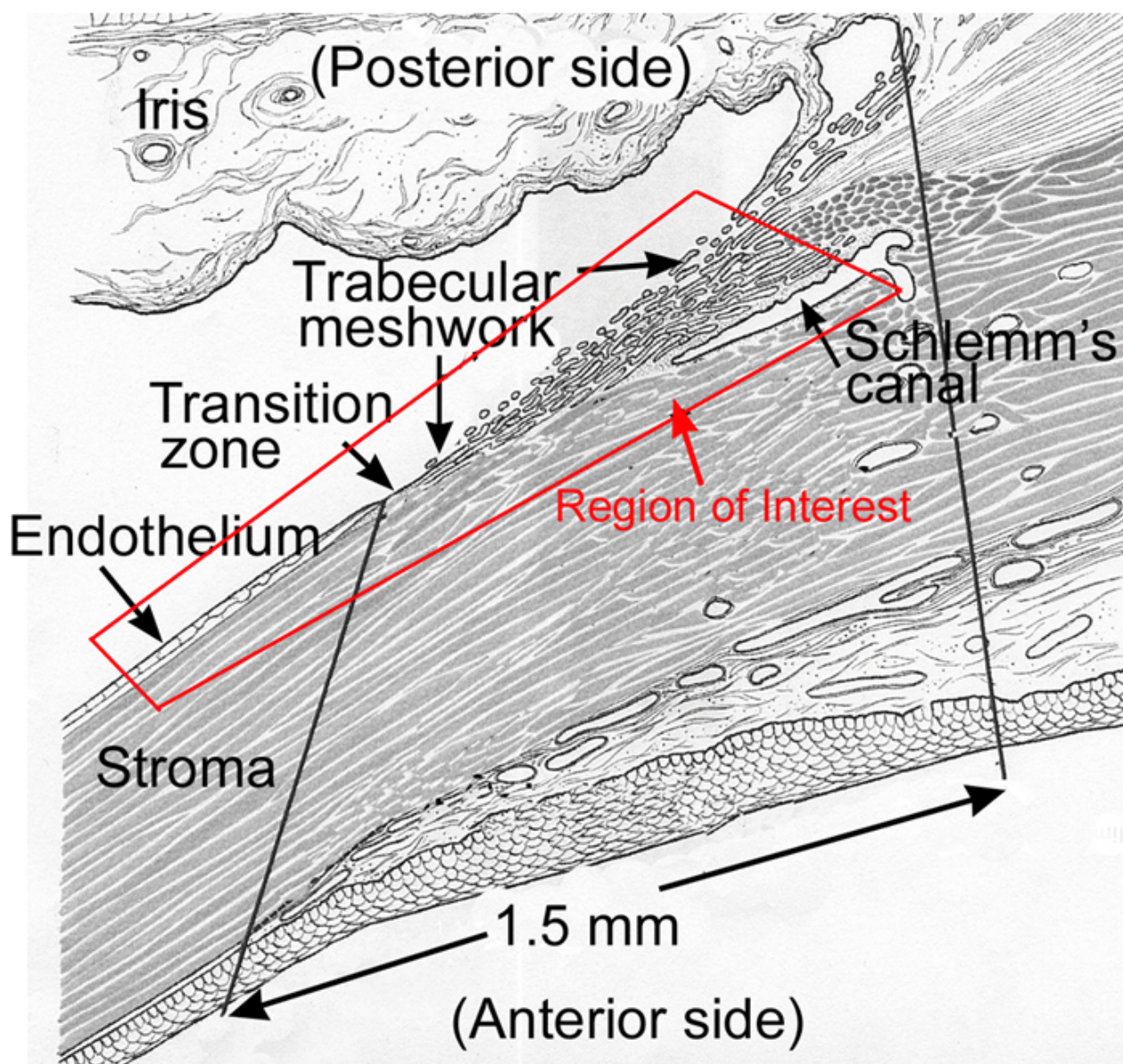


Figure 1. Transverse diagram of the human cornea and limbus. The diagram illustrates the anatomic relationships of the tissues and regions discussed in this study (outlined in red). The posterior limbus is the region between the solid vertical lines as shown, consisting of the transition zone, the trabecular meshwork, and Schlemm's canal. The corneal endothelium is not part of the limbus. Adapted from Histology of the Human Eye, Hogan, Alvarado, Weddell, The Limbus, p.113, 1971, with permission from Elsevier.

TABLE 1. PRIMARY ANTIBODIES						
Antibody	Description	Source/ catalog ID	Concentration (µg/ml)			
			Cross sections		Flat mounts	
			Primary	Secondary	Primary	Secondary
Nestin	mouse, monoclonal	Abcam* ab6320	0.1	0.5	0.03	0.2
Oct-3/4	rat, monoclonal	R&D# MAB1759	0.5	1.0	0.1	0.2
Pax-6	rabbit, polyclonal	Abcam* ab5790	1.3	0.2	0.2	0.2
Sox-2	mouse, monoclonal	R&D# MAB2018	0.5	1.0	0.5	0.2
Telomerase	rabbit, monoclonal	Abcam* ab32020	0.75	1.3	0.6	0.4
Wnt-1	rabbit, polyclonal	Abcam* ab15251	0.25	1.3	0.07	0.2

Below is a list of primary antibodies used for protein detection together with their animal sources, commercial sources and concentrations. Working concentrations of the corresponding secondary antibodies are listed here for convenience. Secondary antibody descriptions are listed in Table 2. All antibodies were used as described in the Methods section. The asterisk indicates Abcam, Cambridge, MA and the sharp (#) indicates R&D Systems, Minneapolis, MN.

TABLE 2. SECONDARY ANTIBODIES			
Antibody	Conjugation	Absorption/ emission	Catalog number
Rabbit anti-Mouse IgG	Texas Red	596 nm/620 nm	ab6726
Goat anti-Mouse IgG	FITC	495 nm/528 nm	ab6785
Sheep anti-Rabbit IgG	Texas Red	596 nm/620 nm	ab6793
Rabbit anti-Rat IgG	TRITC	550 nm/570 nm	ab6731

Below is a list of secondary antibodies used for immunofluorescence together with their description, conjugation, absorption, and emission wavelengths and catalog numbers. All secondary antibody dilution information is listed in Table 1. All antibodies were used as described in the Methods section. All antibodies were from Abcam, Cambridge, MA. In the table, “Texas Red” is Texas Red sulfonyl chloride, “FITC” is Diaminotriazinylaminofluorescein, and “TRITC” is Tetramethylrhodamine isothiocyanate.

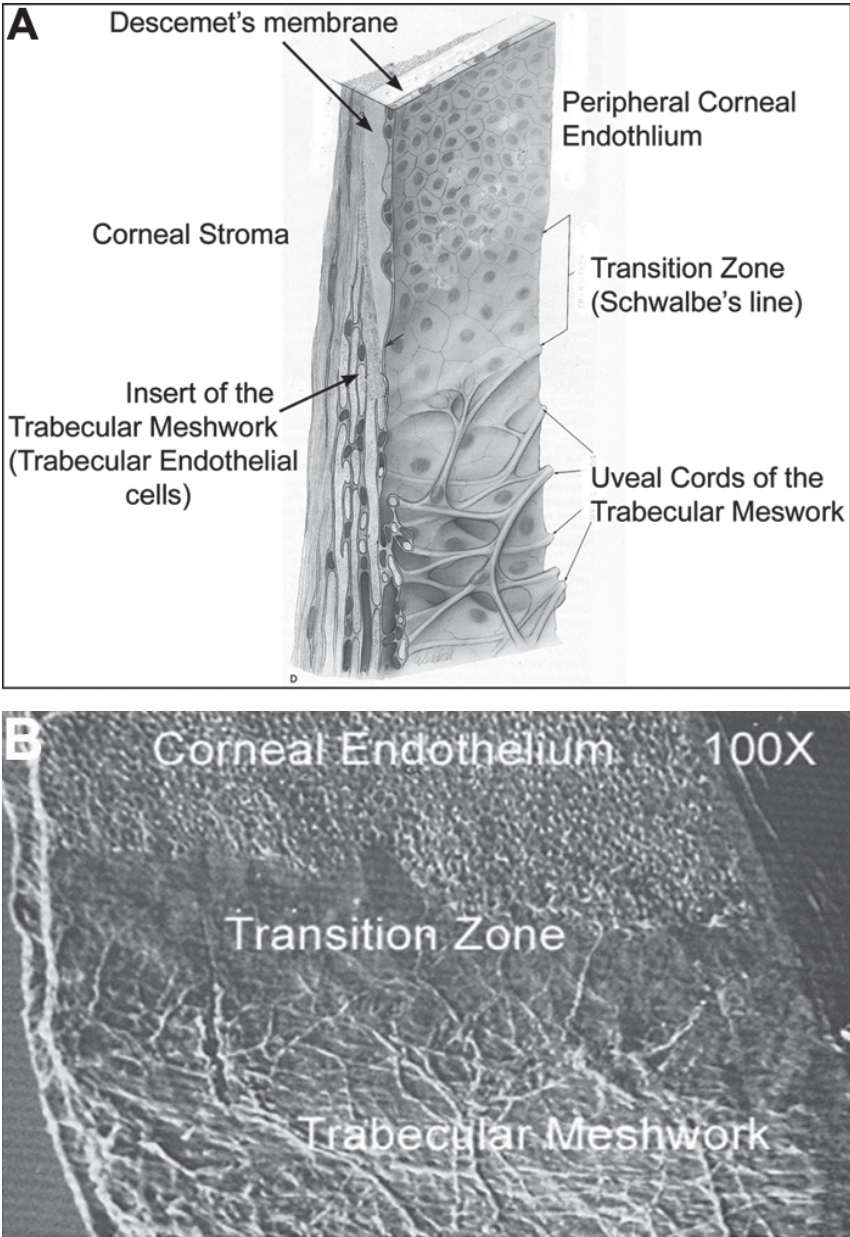


Figure 2. Three-quarter and flat mount views of the human cornea and limbus. The diagram at the top (A) illustrates the anatomic relationships of the tissues and regions discussed in this study. The area indicating the insert of the trabecular meshwork includes cells at the border of Schlemm’s canal. A scanning electron micrograph of a human corneal flat mount is shown in B. Adapted from Histology of the Human Eye, Hogan, Alvarado, Weddell, The Limbus, p. 176-177, 1971, with permission from Elsevier.

zen Tissue Embedding Media (Fisher Scientific, Pittsburgh, PA). Ten micrometers of transverse sections were made to ensure the integrity of the trabecular meshwork. After 24 h of drying, slides were either stained immediately or stored at -20 °C.

Preparation of Flat Mounts: Whole, fixed corneas were cut in half and stored in PBS. In preparation for staining, incisions were made around the periphery of the corneas to allow them to lay flat for mounting and viewing on a glass slide.

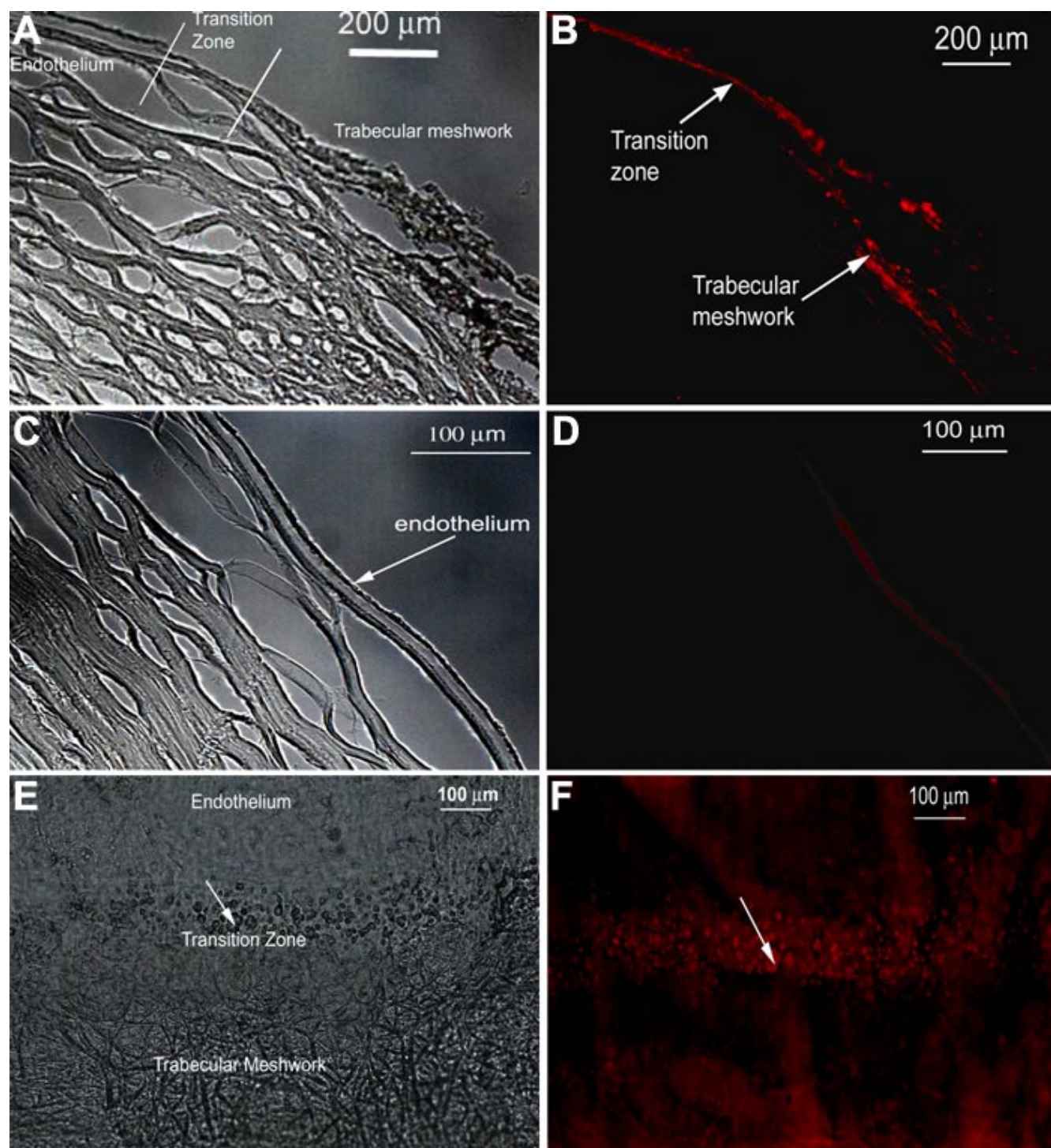


Figure 3. Nestin in the unwounded cornea. Brightfield image (A) and corresponding fluorescence image (B) shows the presence of nestin in the trabecular meshwork and the transition zone of a cross section of the unwounded cornea. Nestin is not present in the corneal endothelium although there is some autofluorescence indicated at the border of the stroma and Descemet's membrane (D); corresponding brightfield image (C). Sections are from a 20-year-old male. Flat mount of an unwounded cornea shows nestin in the transition zone with an arrow indicating the same cells in the corresponding panels (E, F). Flat mount cornea is from a 64-year-old female. Controls showed no reaction (not shown).

Immunohistochemistry: Sectioned or whole tissues were treated with a blocking solution in PBS containing donkey serum (1% for whole tissue, 5% for sections), 0.1% Triton X-100, and 1% BSA for 1 h at room temperature. The tissue was incubated with diluted primary antibody overnight at 4 °C or for 1 h at 37 °C. Table 1 lists the primary antibodies that were used and their concentrations. Antibodies were diluted in Incubation Buffer-TX (66 mM Tris-HCl, 0.66 mM MgCl₂, 1 mM 2-mercaptoethanol, and 0.1% Triton X-100, pH 7.4).

Primary antibodies for nestin, telomerase, Oct-3/4, Pax-6, Sox-2, and Wnt-1 were used with appropriate fluorescein-conjugated secondary antibodies as listed in Table 2. Antibodies were diluted further for use with flat mount preparations to reduce background. After treatment with secondary antibody, some tissues were exposed to a 1 µg/ml solution of Hoechst 33342 (Molecular Probes, Carlsbad, CA) nuclear stain for 15 min. Tissues were washed with PBS between all steps of the staining procedure. The tissues were mounted on glass slides with Gel/Mount, anti-fade medium (Biomed, Burlingame, CA) and were allowed to set overnight at room temperature.

Alkaline Phosphatase: Stem cell specific, alkaline phosphatase activity was determined using an Enzyme-Labeled Fluorescence-97 (ELF) Phosphatase Detection Kit (American Type Culture Collection, Manassas, VA) according to the manufacturer's protocol and as described by Cox and Singer [14]. Briefly, fixed sections or corneal halves for flat mounts

were permeabilized in 0.2% Tween-20 in PBS for 10 min at room temperature and rinsed in PBS. The alkaline phosphatase substrate: ELF [2-(5'-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone] was diluted in detection buffer 30 fold, and the solution was filtered through a 0.2 µm filter just before applying to tissue. The substrate solution was applied at the microscope, and the reaction was viewed at 100X with a DAPI filter (4'6-diamidino-2-phenylindole; excitation 365±8 nm; emission greater than or equal to 400 nm). The reaction was stopped by submerging the tissue in a wash buffer of PBS with 25 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM levamisole, pH 8.0. Average reaction times were 30-90 s. The tissues were mounted with ELF-97 mounting medium (provided in the kit), allowed to dry overnight and photographed.

Controls: Controls were performed with each assay. For immunohistochemistry, these consisted of a sample not exposed to antibody, a sample exposed to only the primary antibody, a sample exposed to only the secondary antibody, and a brightfield examination of the sample exposed to both antibodies. In some cases, the Hoechst stain (previously described) was used for the location of cell nuclei. The control for the alkaline phosphatase assay consisted of a sample not exposed to the ELF substrate.

Photography: Slides were viewed at 100X, 200X, and 400X magnifications using fluorescence microscopy (Zeiss, ApoCam, AxioVision 4.5, Jena, Germany). The focus level

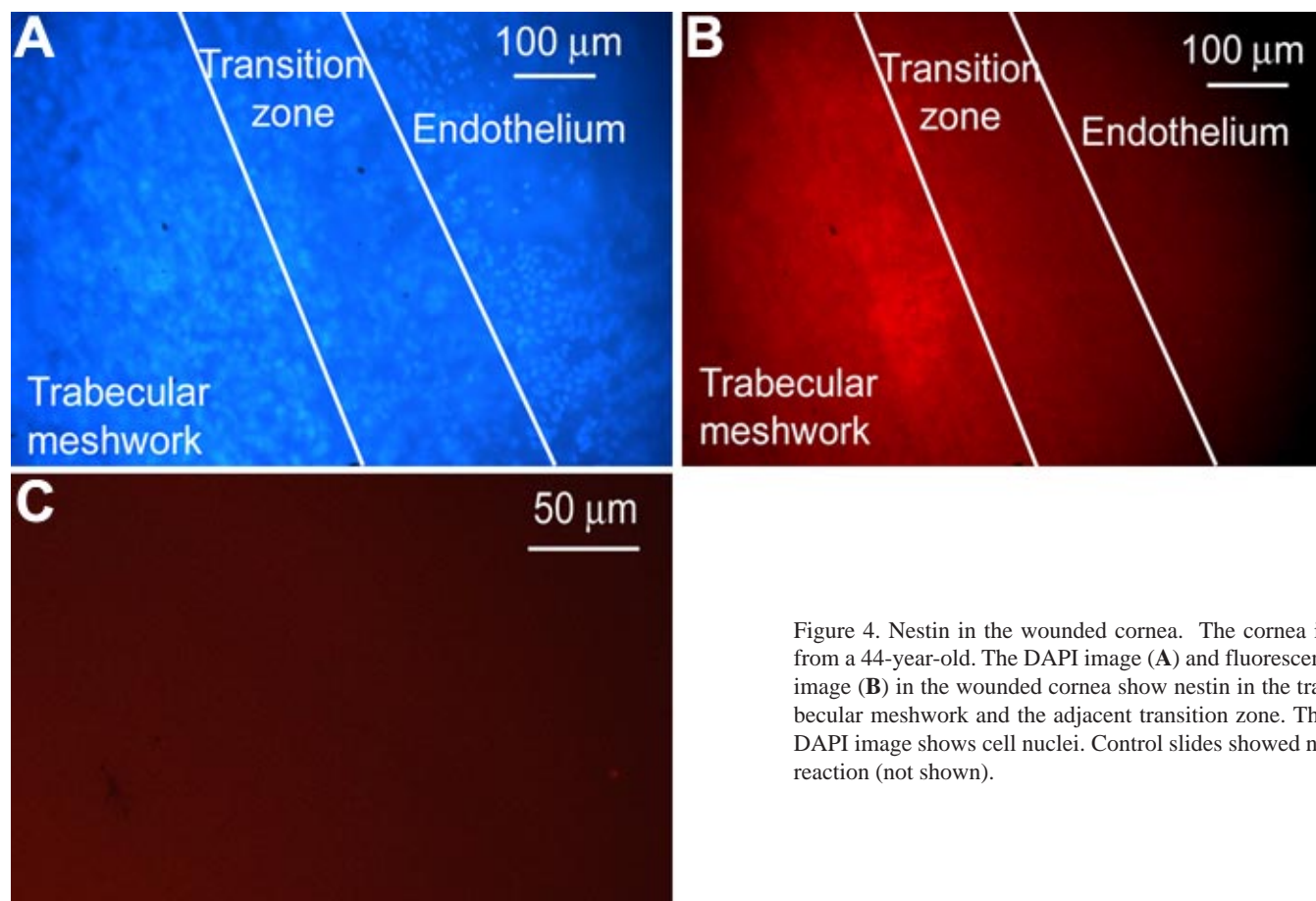


Figure 4. Nestin in the wounded cornea. The cornea is from a 44-year-old. The DAPI image (A) and fluorescent image (B) in the wounded cornea show nestin in the trabecular meshwork and the adjacent transition zone. The DAPI image shows cell nuclei. Control slides showed no reaction (not shown).

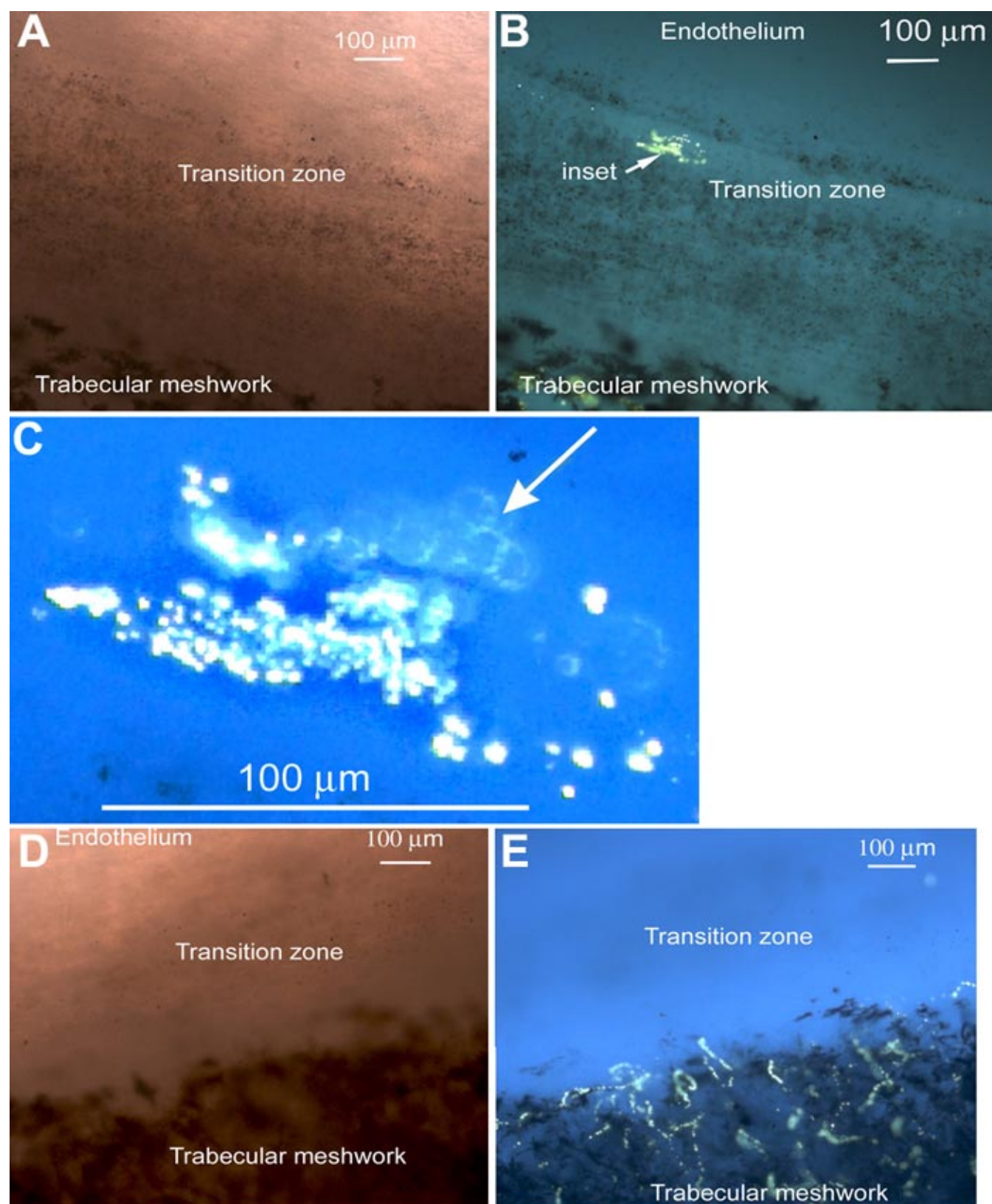


Figure 5. Alkaline phosphatase in the unwounded cornea. The cornea is from a 33-year-old male. Brightfield image (A) and alkaline phosphatase activity at Schwalbe's line (B) with an enlarged image (C) is shown. The arrow in C indicates individual cell outlines. Alkaline phosphatase activity in the trabecular meshwork is shown in D and E. All images shown are flat mounts. Results are similar in the wounded cornea while the controls showed no reaction (neither shown).

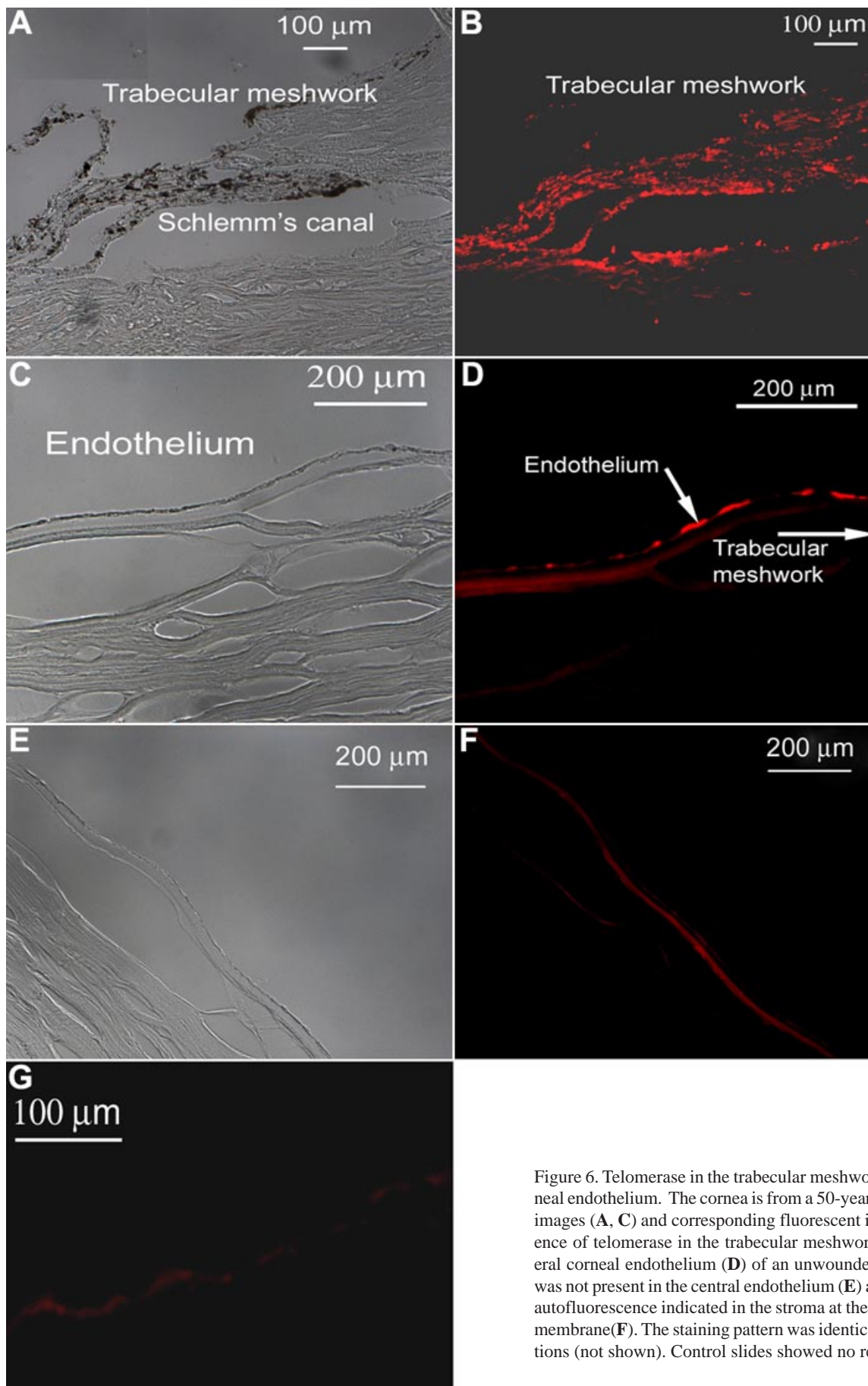


Figure 6. Telomerase in the trabecular meshwork and peripheral corneal endothelium. The cornea is from a 50-year-old male. Brightfield images (A, C) and corresponding fluorescent images show the presence of telomerase in the trabecular meshwork (B) and the peripheral corneal endothelium (D) of an unwounded cornea. Telomerase was not present in the central endothelium (E) although there is some autofluorescence indicated in the stroma at the border of Descemet's membrane(F). The staining pattern was identical in the wounded sections (not shown). Control slides showed no reaction (G).

was not adjusted between brightfield and fluorescent images. Test slides were viewed and digitally photographed to determine the optimal exposure time. Control slides were photographed without adjusting the exposure time.

RESULTS

Nestin: Fluorescent staining for nestin occurred at the trabecular meshwork and the transition zone in both unwounded (Figure 3) and wounded corneas (Figure 4). In the unwounded corneas, nestin labeling was pronounced in the transition zone and the trabecular meshwork as seen in transverse sections (Figure 3B). Although seeming to occur at endothelial cells, this was observed to be autofluorescence as shown in Figure 3D. Fluorescence was also quite marked around the circumference of the transition zone as seen in flat mounts (Figure 3F). In wounded corneas, labeling was observed in regions of the trabecular meshwork and the adjacent transition zone (Figure 4B). However, the cells were less well delineated possibly due to the multiple layers of cells in the flat mount. Controls

that only received a secondary antibody did not show any labeling as shown in Figure 4C.

Alkaline phosphatase: Alkaline phosphatase activity in the unwounded cornea was detected in both the trabecular meshwork and in discrete regions of the transition zone (Figure 5). Figure 5B shows staining for the transition zone and the trabecular meshwork (lower left hand corner). An enlargement of Figure 5B, shown in Figure 5C at the transition zone, indicates some cell outlines (indicated by an arrow). The results for wounded corneas were similar. Controls that were not exposed to the substrate did not indicate any fluorescence. Figure 5E indicates a larger area of the trabecular meshwork and shows fluorescence due to alkaline phosphatase activity.

Telomerase: Telomerase was present in the trabecular meshwork, transition zone, and peripheral endothelium of both unwounded and wounded corneas. This labeling can be clearly seen in the trabecular meshwork and borders of Schlemm's canal in Figure 6B. It is also seen at the boundary of the endothelium in Figure 6D but not at more central endothelial

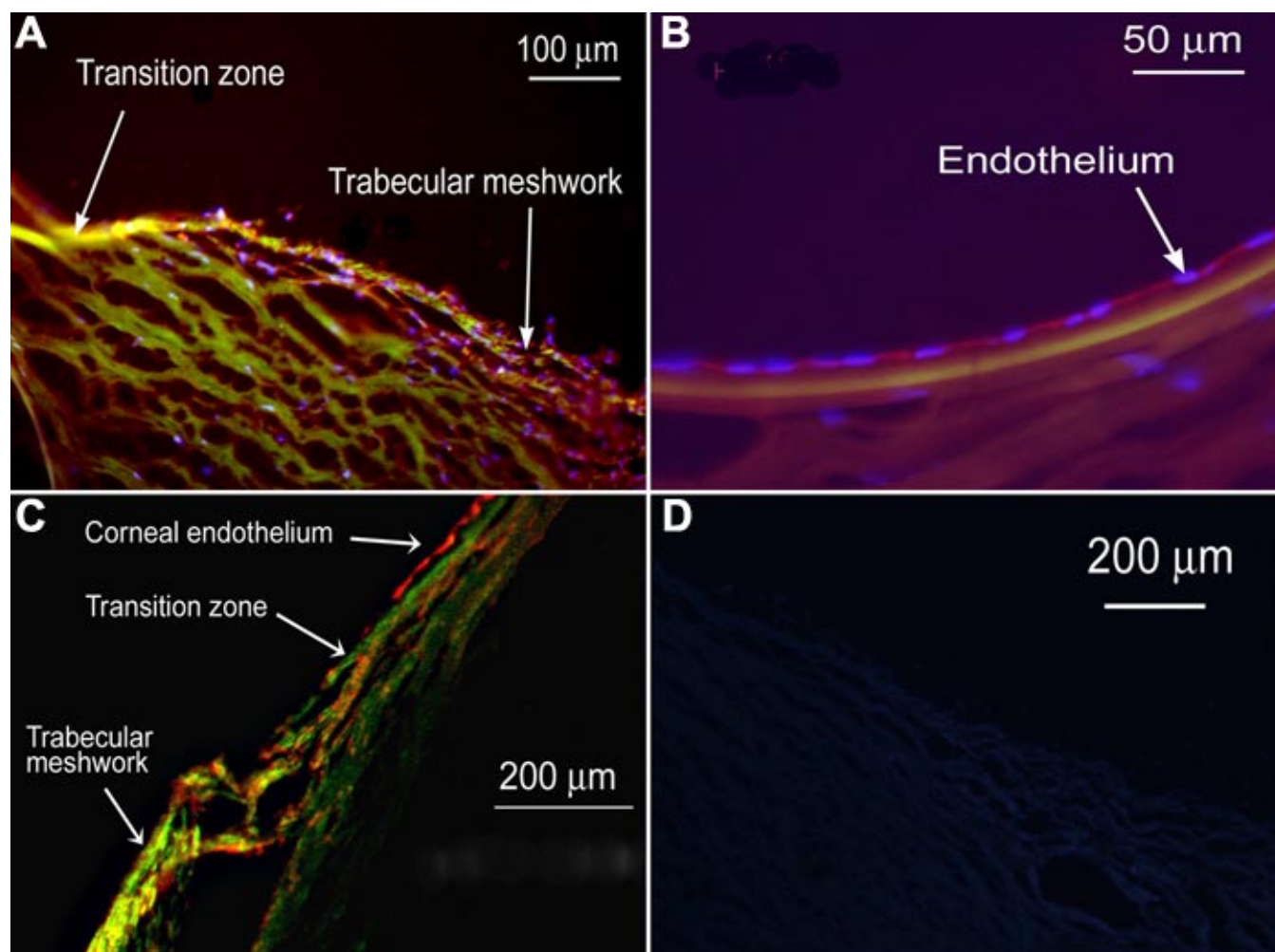


Figure 7. Double labeling: nestin and telomerase. In the unwounded (A, B) and wounded (C) corneal sections, nestin is present in the trabecular meshwork and the transition zone, and telomerase is present in the trabecular meshwork, transition zone, and the corneal endothelium. Bright green indicates nestin; red indicates telomerase; and blue indicates cell nuclei from Hoechst stain. All sections shown are from a 38-year-old male. Control slides showed no reaction (D).

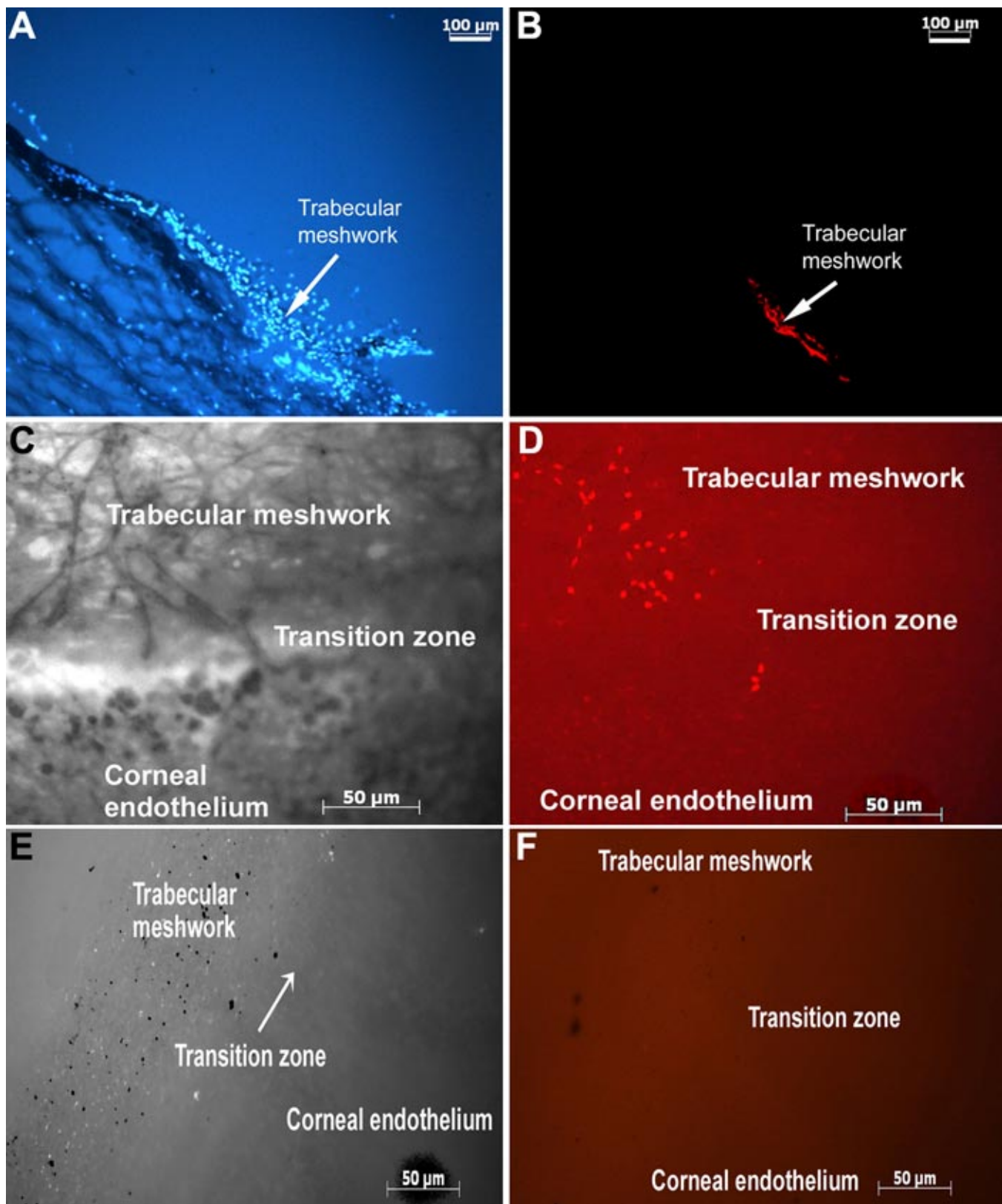


Figure 8. Oct-3/4 in the trabecular meshwork after corneal wounding. DAPI image (A) and brightfield image (C) with corresponding fluorescent images (B, D) shows that Oct-3/4 is present in the trabecular meshwork of a wounded cornea. Corneal cross sections are from a 25-year-old male, and flat mounts are from a 43-year-old male. Oct-3/4 was not present in the unwounded cornea of a 27-year-old male (E, F). A and B are cross sections. C-F are flat mounts. Control slides showed no reaction (not shown).

regions (Figure 6F). The hazy fluorescence in Figure 6D,F represents an undefined autofluorescence in the most posterior part of the stroma. This may also be seen in a control of secondary antibody only in Figure 6G. Figure 7 shows the results of double labeling for nestin (bright green) and telomerase (red). Here, the labeling of nestin can be seen in the transition zone in Figure 7A. Telomerase, however, seems to be located a little deeper in the tissue as seen in Figure 7A,C (unwounded and wounded, respectively). The double labeling of wounded and unwounded cornea sections with nestin and telomerase indicates that both of these markers are equally present in the trabecular meshwork. Controls exposed only to secondary antibody did not indicate any labeling (Figure 7D).

Oct-3/4: Oct-3/4 was only indicated in the wounded corneas and primarily in the trabecular meshwork. In cross sections of the wounded corneas, Oct-3/4 was found only in the trabecular meshwork (Figure 8B). However, when viewed as a flat mount, the marker also seemed to occur in a few isolated regions of the transition zone in addition to the trabecular meshwork (Figure 8D). There was no staining in the unwounded cornea (Figure 8F), and no staining was seen in controls that were exposed only to the secondary antibody.

Wnt-1: In the wounded state, Wnt-1 staining occurred heavily in the trabecular meshwork particularly around Schlemm's canal and secondarily at the endothelial border (Figure 9B). Staining was not seen with an unwounded cornea (Figure 9D) and did not occur with controls exposed only

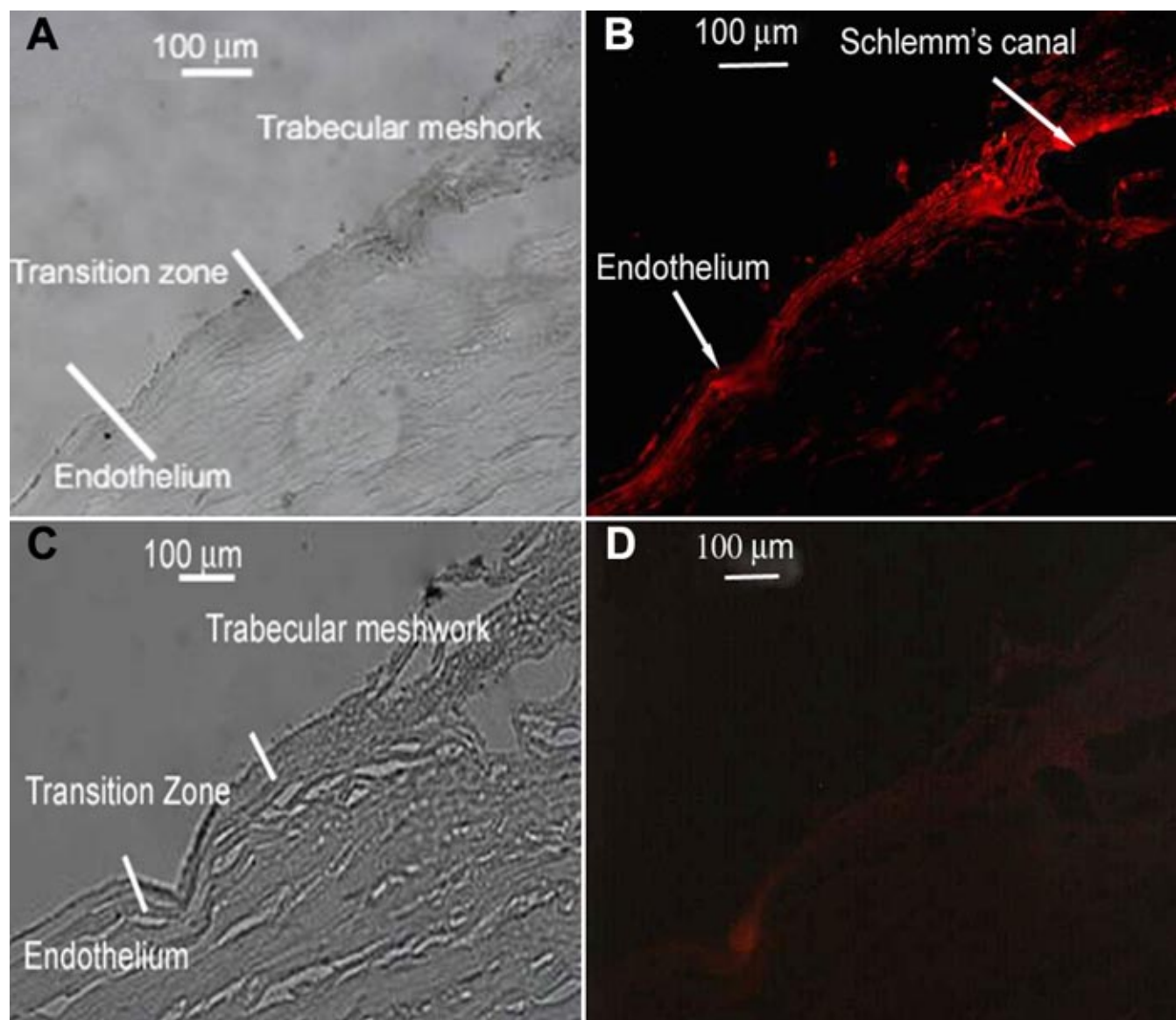


Figure 9. Wnt-1 in the wounded cornea. Cornea is from a 50-year-old male. Brightfield image (A) and corresponding fluorescent image (B) showing Wnt-1 presence in the trabecular meshwork, particularly the endothelial border of Schlemm's canal of a wounded cornea. Wnt-1 is not present in the trabecular meshwork of a 38-year-old whose cornea was unwounded. (C, D). All images are cross sections. Control slides showed no reaction (not shown).

to the second antibody for either wounded or unwounded tissues.

Pax-6: *Pax-6* staining only occurred after wounding. As shown in Figure 10B,D, the presence of *Pax-6* was indicated

in the trabecular meshwork, the transition zone, and the peripheral endothelium. However, as indicated in Figure 10F, *Pax-6* was not seen with unwounded corneas. Figure 11 indicates that double labeling of wounded corneas with nestin and

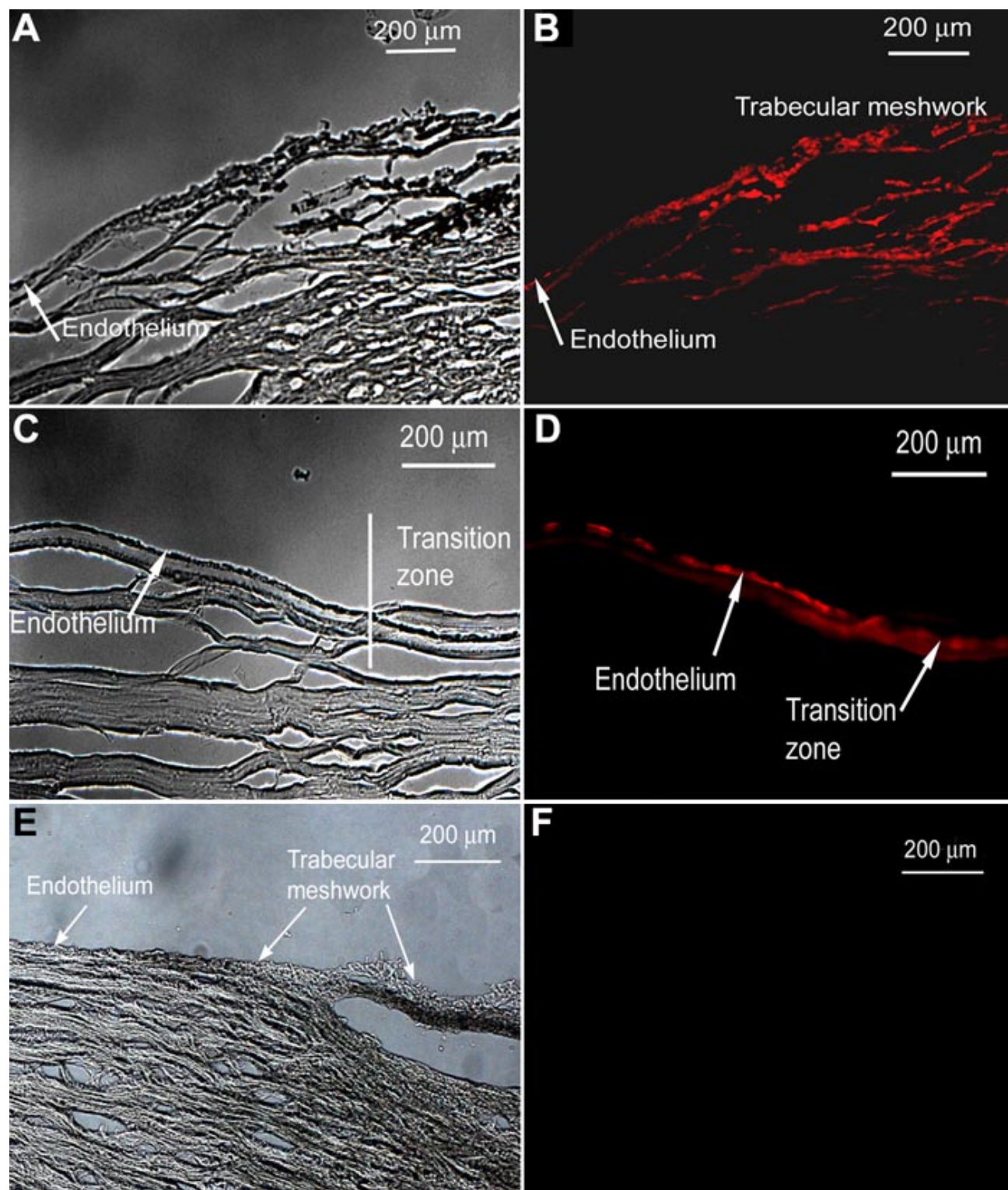


Figure 10. *Pax-6* in the wounded cornea. The cornea is from a 38-year-old male. Brightfield images (A, C) and corresponding fluorescent images (B, D) show the presence of *Pax-6* in the trabecular meshwork and the corneal endothelium of a wounded cornea. *Pax-6* is not present in unwounded tissue from a 29-year-old male (E, F). All images are cross sections. Control slide showed no reaction (not shown).

Pax-6 indicated a strong presence of Pax-6 in the cells surrounding Schlemm's canal and the trabecular meshwork. Nestin was strongly present in the trabecular meshwork as well. Pax-6 was not present in the unwounded corneal sections with double labeling (Figure 11B).

Sox-2: After wounding, Sox-2 was present in the endothelium, transition zone, and the trabecular meshwork as seen in Figure 12B. Sox-2 labeling, however, was not seen in the unwounded cornea (Figure 12D).

Summary of labeling data: In the unwounded cornea, the stem cell markers nestin, alkaline phosphatase, and telomerase

were present in the trabecular meshwork and at the transition zone. Only telomerase was present in the peripheral endothelium. After corneal wounding, labeling of the three stem cell markers (nestin, telomerase, and alkaline phosphatase) remained as before. However, wounding also produced the appearance of the stem cell markers Oct-3/4 and Wnt as well as the differentiation markers Pax-6 and Sox-2. All these results are summarized in Table 3 and Figure 13.

DISCUSSION

Markers in the posterior limbus with an unwounded corneal endothelium: Three stem cell markers were found in the posterior limbus when the cornea was unwounded: nestin, alkaline phosphatase, and telomerase. All three markers are established markers for stem cells.

Nestin, an intermediate filament protein, is expressed in neuroepithelial stem cells during embryonic development including neuroectodermal stem cells and neural crest cells [15-17]. It is well known that neural crest cells are precursor cells for the corneal endothelium. It was not surprising that the cell population in the trabecular meshwork and the transition zone between the periphery of the cornea and the trabecular meshwork expressed this protein. The development of corneal endothelial tissues is reviewed in [18].

Alkaline phosphatase is an enzyme whose activity also identifies pluripotent stem cell populations. Such alkaline phosphatase activity would be downregulated in differentiated cells [19]. Multiple isoforms of this enzyme exist, and the form that indicates stem cell presence is identical to that produced in the bone, liver, and kidney [20]. It is the isoform that was found throughout the trabecular meshwork and in isolated locations of Schwalbe's line cells. The Schwalbe's line expression pattern suggests that the majority of stem cells are stored in the trabecular meshwork while pockets of stem cells exist in niches outside of the periphery of the endothelium. The cells in the transition zone (Schwalbe's line) seem to be cells that are used as transient amplifying cells in the corneal endothelial periphery.

Cells in the posterior limbus were also found to express telomerase, an enzyme that preserves chromosomal codes by maintaining telomere lengths [21]. Telomerase presence and activity are characteristic of progenitor cells and are considered markers for both stem cells and for transient amplifying cells [22,23]. The presence of telomerase also suggests that there are two populations of progenitor cells: a stem cell population in the trabecular meshwork and a population of transient amplifying cells in the transition zone to be used for the corneal endothelium. The presence of telomerase in the peripheral endothelium is consistent with reports that corneal endothelial cells divide and spread into the central endothelium during a wounding response [12,24-26].

Additional markers found in the posterior limbus and the endothelium after wounding: The appearance of additional markers in both the posterior limbus and the endothelium after wounding indicate that stem cells in these tissues are activated and mobilized to sites where differentiated endothelial cells are needed.

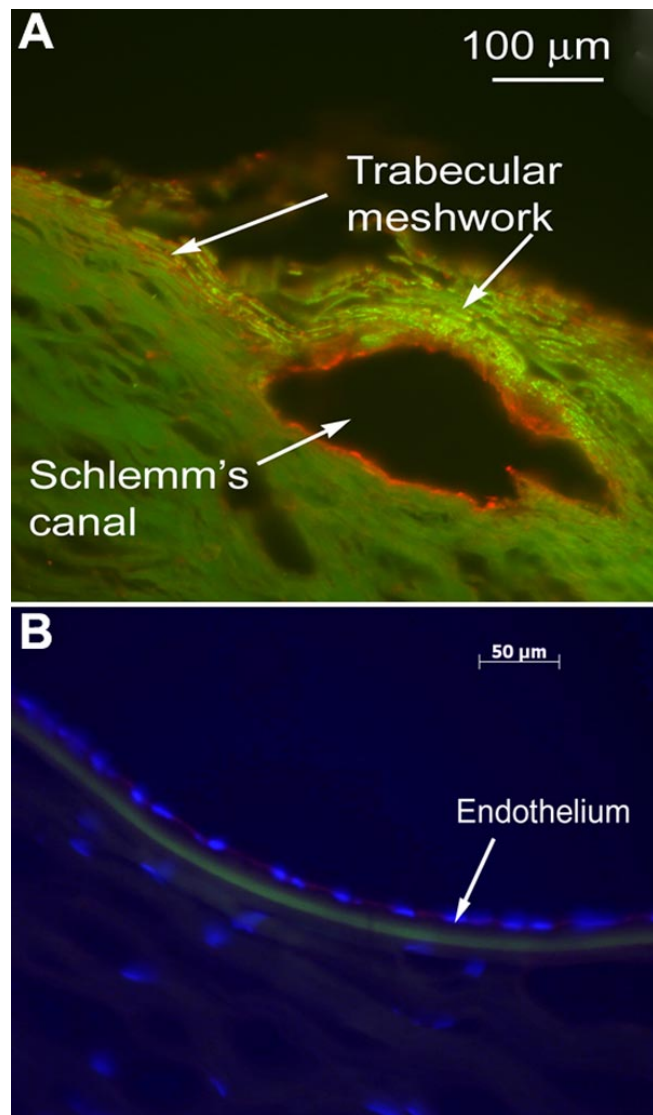


Figure 11. Double labeling: nestin and Pax-6. The wounded cornea is from a 38-year-old male. Pax-6 (red) was present in the endothelium, transition zone, and the interior border cells of Schlemm's canal, and nestin (bright green) is present in the trabecular meshwork and at the transition zone (A). Double labeling in the unwounded cornea of 29-year-old male shows no expression of Pax-6 (B). Bright green indicates nestin; red indicates Pax-6; and blue indicates cell nuclei from Hoechst stain. Control slide showed no reaction (not shown).

The presence of Oct-3/4 was an indication of the persistent residence of primitive stem cells [27-29] in the trabecular meshwork, and the protein may represent a signal for symmetric cell division of only those stem cells that reside in that tissue. Historically, Oct-3/4 has been shown to maintain the high proliferative capacity of early stem cells [30,31]. As shown in Figure 14, the committed daughter cells may move from the region of Oct-3/4 expression to the outer portion of the trabecular meshwork and then into the transition zone and eventually to the endothelial layer (Pathway 1). Another possibility is that these cells migrate to the periphery of Schlemm's canal and then into the transition zone before entering the endothelium (Pathway 2). The cells at the periphery of Schlemm's canal have been described as endothelial insert cells [32]. While these cells move through the adjacent tissues/niches, they seem to express the differentiation proteins as observed in this study. It is suggested, then, that there are two niches for these cells, one in the trabecular meshwork and the other in the transition zone (including Schwalbe's line).

Pax-6 codes for a DNA binding domain, which is referred to as paired domain [33,34] or paired-like homeodomain transcription factor [35]. It is a protein involved in many transcription events including those that facilitate the transport of neural crest cells. Cvekl and Tamm [18] have carefully reviewed how this transcription factor is related to early eye development. They further explain the need of this factor for differentiation of new cells such as those required after wounding. Indeed, either the lack of Pax-6 or its mutation causes aniridia [36] and glaucoma in humans. Corneal development is impaired as well. Repair is assumed to be a maintenance function of development.

Sox-2 is a member of a family of transcription factors [37] that are expressed in a variety of both embryonic and adult tissues [38]. The importance of this family of proteins is indicated in Sox-2 null mutant mouse embryos who do not survive past implantation [39]. Sox-2 and other Sox factors are also expressed in neural crest cells. Sox-2 functions to maintain pluripotent cells and neural stem cells [39] as well as the

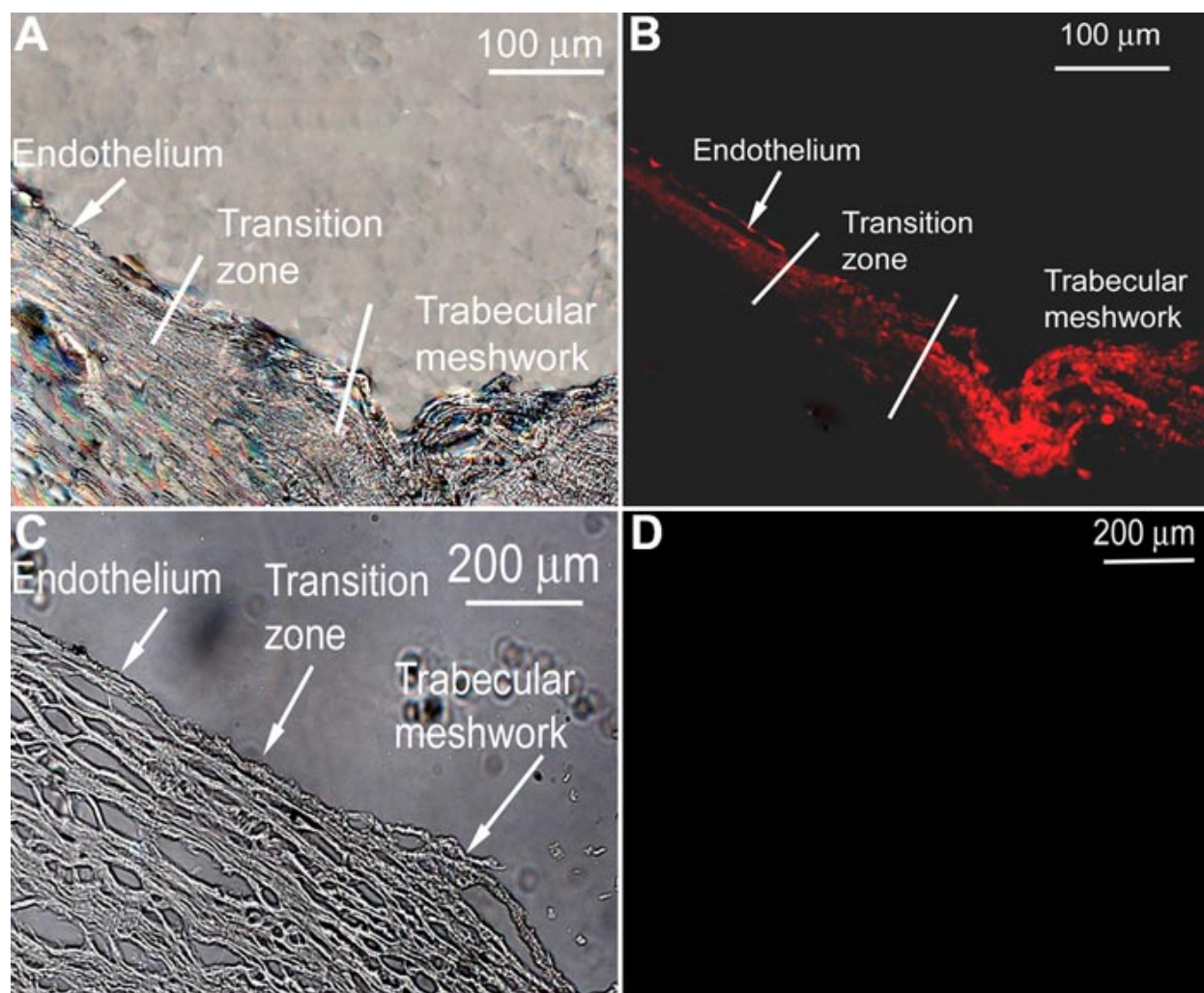


Figure 12. Sox-2 in the trabecular meshwork, transition zone, and corneal endothelium after wounding. The brightfield image (A) and the corresponding fluorescent image (B) show the presence of Sox-2 in the trabecular meshwork, transition zone, and corneal endothelium of a wounded cornea from a 38-year-old male. Sox-2 is not present in the unwounded cornea of a 29-year-old male (C, D). All images are cross sections. Control slides showed no reaction (not shown).

adult nervous system [37]. Its role in the adult (as shown in this study) is more likely to be concerned with cell differentiation rather than as a marker for non-dividing stem cells [37]. This would mean its presence is required for the asymmetric division of stem cells.

Wnt proteins is also found after wounding in this study. They are highly conserved, lipid-modified growth factors that regulate cell proliferation and cell fate in multiple tissues of both the developing embryo and adults [40-45]. However, the expression pattern of Wnt-1 in this study suggests that similar to Pax-6 and Sox-2, it has a role in the maturation of cells in response to wounding and therefore may also demonstrate a stem cell population that is undergoing asymmetric cell division.

All of this data support work by Treffers [24], Olsen [25], and Laing et al. [26] in which the investigators showed the mitosis and migration of cells from the periphery into the wounded areas of the corneal endothelium to repair damaged tissue. A previous study [12] is also supportive in that it re-

ports telomerase activity is found at the peripheral endothelium in unwounded tissues. Another report [46] suggests that stem cells can be found within the corneal endothelium. The stem cells were reported to reside in neurospheres of managed cultures of human corneal endothelium. However, those cells may represent corneal endothelial cells that have dedifferentiated into a small percentage of stem-like cells.

Embryological considerations and Schlemm's canal endothelial cells: Since the corneal endothelium originates from the mesenchymal branch known as the neural crest [47], the findings of this study have interesting implications for the relationship between the posterior limbus and the corneal endothelium. Embryologically, corneal endothelial cells first migrate into the endothelial area in loose layers ([48,49] and as reviewed in [18]). The cells condense into a monolayer forming the corneal endothelium. The remaining mesenchymal cells form into the fibroblasts and keratocytes of the corneal stroma [48-50]. The trabecular meshwork develops after these events, and its cells form a continuous cell layer with the corneal en-

TABLE 3. SUMMARY OF POSITIVE AND NEGATIVE RESULTS OF ALL MARKERS

Marker/ preparation method	Number of corneas evaluated	Unwounded cornea	Wounded cornea (24-48 h)
Nestin	16		
Cross-section		TM, Schwalbe's line	TM, Schwalbe's line
Flat mount		Schwalbe's line	Schwalbe's line
Alkaline Phosphatase	6		
cross-section		TM	TM
flat mount		TM, Schwalbe's line	TM, Schwalbe's line
Telomerase	10		
cross-section		TM, Schwalbe's line, peripheral endothelium	TM, Schwalbe's line, peripheral endothelium
flat mount		no reaction	TM
Oct-3/4	8		
cross-section		no reaction	TM
flat mount		no reaction	TM
Wnt-1	8		
cross-section		no reaction	TM, Schwalbe's line
flat mount		no reaction	no reaction
Pax-6	7		
cross-section		no reaction	TM, Schwalbe's line, peripheral endothelium
flat mount		no reaction	no reaction
Sox-2	2		
cross-section		no reaction	TM, Schwalbe's line, endothelium
flat mount		no reaction	no reaction

Below is a list of all molecular markers used along with the total number of corneas evaluated for each marker and the results seen in unwounded and wounded cornea for each given preparation method, in either cross-section or flat mount. In the table, "TM" indicates trabecular meshwork.

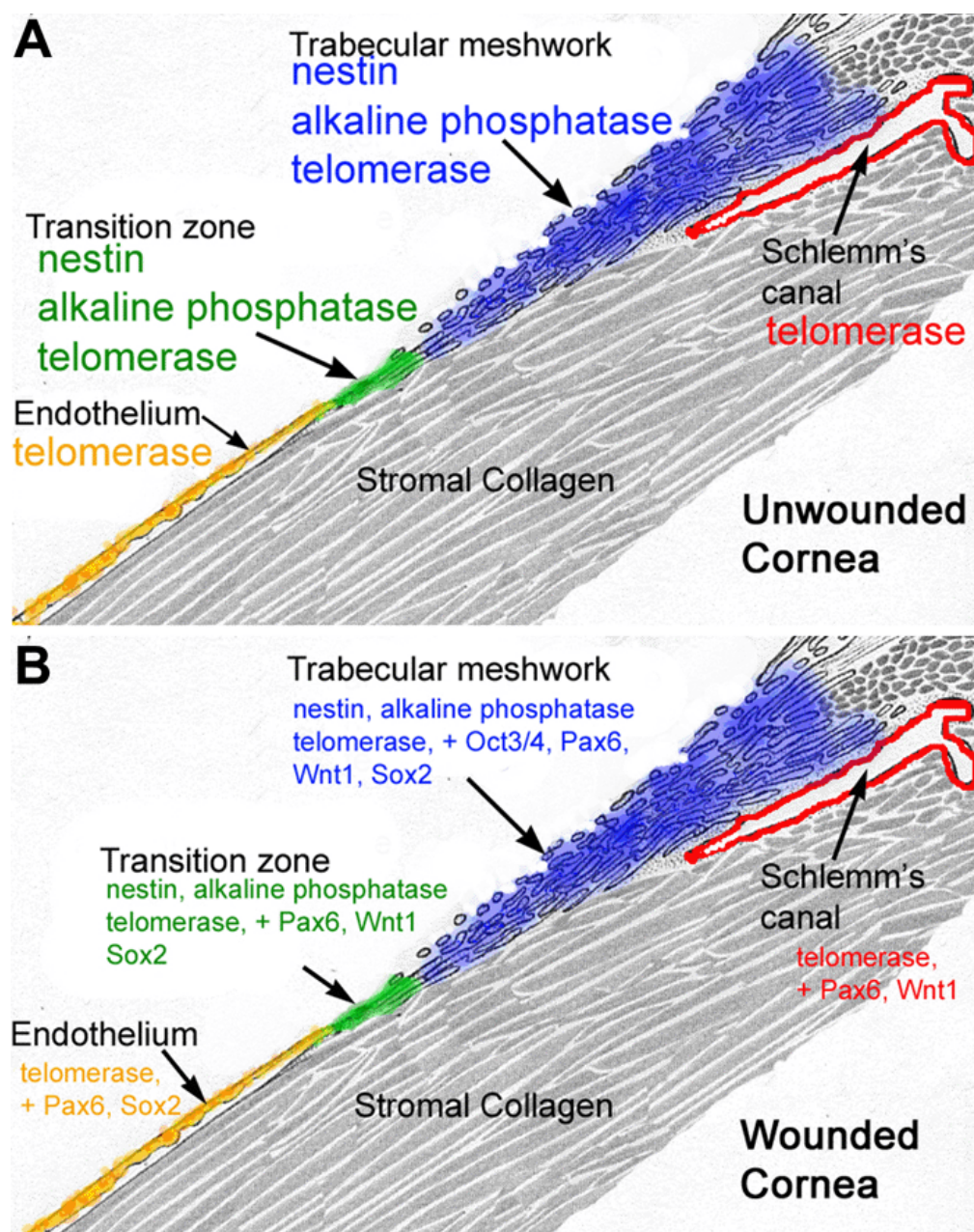


Figure 13. Results diagrams. Locations of antibody staining in the unwounded (**A**) and the wounded cornea (**B**) are shown. The (+) in the diagram of the wounded cornea indicates those markers that appeared following wounding.

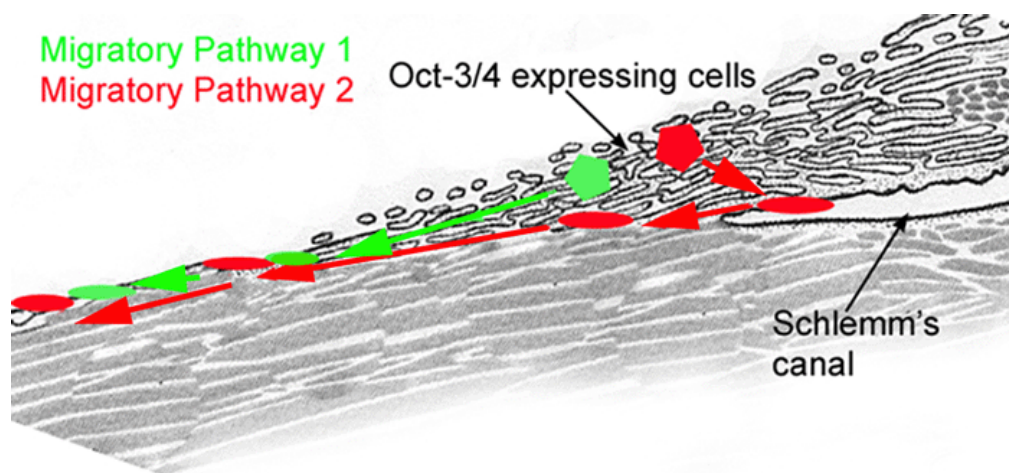


Figure 14. Possible migratory pathways for Oct-3/4 expressing cells. Cells may proceed from the region of Oct-3/4 expression to the transition zone and into the corneal endothelium (1) or through the trabecular endothelial cells, the transition zone, and into the corneal endothelium (2).

dothelium [51-54]. A second mesenchymal branch, the mesoderm, makes a contribution to the trabecular meshwork area in the form of endothelial-like cells associated with Schlemm's canal [55,56]. Some of these cells insert themselves into the anterior portion of both the transition zone and the outer edge of the corneal endothelium. It is significant that these cells also show stem cell markers. Meier [57] has suggested that these Schlemm's canal endothelia give directional cues to neural crest cells during migration. Such a role may continue with the adult for the cells of the trabecular meshwork, the transition zone, and the peripheral edge of the corneal endothelium. This would suggest that migration pathway 2 (Figure 14) is one in which the cells of the trabecular meshwork, the transition zone, and the edges of the corneal endothelium are partly influenced by cytokines originating from the Schlemm's canal endothelia and therefore could represent a second type of stem cell present in the posterior limbus.

In summary, there are at least two populations of stem cells or precursor cells in the posterior limbus: (1) trabecular meshwork cells and (2) transition zone cells. These cells have an origin from mesenchymal neural crest cells. Endothelial insert cells at the border of Schlemm's canal might represent yet another population of stem cells of mesodermal origin. The evidence shown here suggests that trabecular cells migrate into the transition zone (including Schwalbe's line) and enter the corneal endothelium to produce differentiated endothelial cells. The transported cells could represent an intermediate state between a stem cell and a differentiated corneal endothelial cell (i.e. a transient amplifying cell). The data also suggests routes by which these progenitor cells enter the corneal endothelium both normally and in response to wounding.

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