Molecular Vision 2024; 30:107-113 <http://www.molvis.org/molvis/v30/107>
Received 25 April 2023 | Accepted 15 March 2024 | Published 17 March 2024

Microstructure of the corneal endothelial transition zone in different laboratory animals

Jun Seob Lee, So Young Lee, Hee Seung Chin, Na Rae Kim, Ji Won Jung

Department of Ophthalmology and Inha Vision Science Laboratory, Inha University School of Medicine, Incheon, South Korea

Purpose: To compare the microstructure of the corneal endothelial transition zone in different laboratory animals.
Methods: Flat-mount corneas of rabbits, rats, and mice were stained with Alizarin Red S (ARS) and observed using scanning electron microscopy (SEM). The progenitor cell markers p75 neurotrophin receptor (p75NTR), SRY-box transcription factor 9 (SOX9), leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), telomerase reverse transcriptase (TERT), and proliferation marker K_i-67 were examined in the flat-mounted corneas of three laboratory animals using immunofluorescence microscopy.
Results: On flat mounts, proximity to the trabecular meshwork correlated with weaker ARS staining and greater polymorphism of endothelial cells in the transition zone in all animals. On SEM, distinct and smooth structures of the transition zone were negligibly detected in all animals. The endothelial cells in the transition zone had irregular shapes, with less dense, less wavy intercellular junctions, especially in murine corneas, exhibiting unique intercellular cystic spaces. In the transition zone of the rabbit cornea, progenitor cell markers p75NTR, SOX9, Lgr5, TERT, and proliferation marker K_i-67 were expressed, in contrast to those in other murine corneas.
Conclusions: Although the transition zone was not identified clearly, irregular cell morphology and loss of cell–cell contact were observed in all animal corneal endothelial cells. The proliferative capacity and the presence of progenitor cells were confirmed in the transition zone, especially in the rabbit cornea.

The corneal endothelial transition zone is a smooth and annular area that is adjacent to the end of the peripheral endothelium and lacks trabecular meshwork fiber [1]. The cells of the human corneal endothelial transition zone overlap and exhibit irregular shapes and loss of cell–cell contact [2]. Corneal endothelial density in the transitional zone was significantly higher than in the central cornea when stained with Alizarin red S (ARS) in humans [3,4], and the endothelial periphery and transition zone contained fewer differentiated cells or progenitor cells [4]. These findings suggest the potential proliferative capacity of the endothelial transition zone.

Recent studies have focused on corneal endothelial cell regeneration technology [1,3-9], with some reporting regenerative methods using progenitor cells in the endothelial transition zone [1,8,9]. To study the proliferative capacity of the endothelial transition zone *in vivo* or *ex vivo* in animal models, it is necessary to understand the differences in this zone for each animal species.

Sie et al. summarized studies reporting human corneal endothelial progenitor cells in the endothelial periphery [8]. Among those studies, McGowan et al. observed progenitor cells expressing markers, such as nestin, alkaline phosphatase, and telomerase, in the human transition zone [10], identified the expression of p75 neurotrophin receptor (p75NTR) and SRY-box transcription factor 9 (SOX9) in the human corneal endothelial transition zone [9]. However, studies on corneal endothelial progenitor cells in animals are rare. Nevertheless, the expression of SOX9, leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), nestin, and nerve growth factor receptor (NGFR) has been observed in the corneal endothelial cells of mice at postnatal day three [11]. In bovine eyes aged 1–2 years, stem cell markers of nestin, octamer-binding transcription factor 4, paired box 6, SOX2, and telomerase reverse transcriptase (TERT) were expressed in the endothelial transition zone [12]. Therefore, this study aimed to compare the microstructure of corneal endothelial transition zones and investigate the proliferative capacity and presence of corneal endothelial progenitor cells in three different laboratory animals.

METHODS

Experimental animals: Both eyes of nine New Zealand white rabbits, seven Sprague Dawley® (SD) rats, and nine C57BL/6 mice were used. All the animals were seven weeks old. The rabbits, rats, and mice weighed 1.8–2.0 kg, 200–250 g, and 25–30 g, respectively. Corneal tissues with

Correspondence to: Ji Won Jung, Department of Ophthalmology, Inha University Hospital, 7-206, 3-ga, Shinheung-dong, Jung-gu, Incheon 400-711, South Korea, Phone: 82-32-890-2400, FAX: 82-32-890-2403, email: panch325@gmail.com

a scleral rim were obtained from the enucleated eyes, and the orientation was marked at the time of enucleation. All study protocols followed the Institute for Laboratory Animal Research and the Guide for Animal Experimental Protocol with the approval of the Inha University Institutional Animal Care and Use Committee (INHA-IACUC:200820–710 and 201,103-733).

Observation of flat-mounted corneas: The excised corneas devoid of iris tissue were rinsed with a balanced salt solution (Alcon, Fort Worth, TX). They were then placed in 1% ARS (ScienCell Research Laboratories, Inc., Carlsbad, CA) and dissolved in 0.9% sodium chloride with a pH adjusted to 5.2 for 5 min. After fixation with 4% paraformaldehyde (Biosesang, Seongnam-si, Korea), the tissues were placed in phosphate-buffered saline and shaken to detach the clumping deposit. The nuclei of the corneal endothelial cells were stained with a 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) for 2 min. After a rinse with phosphate-buffered saline for 1 min, the corneas were cut into four radial directions, and their images were observed using a BX43 OLYMPUS optical microscope (Olympus, Tokyo, Japan).

Scanning electron microscopy: The corneas were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and cut in four radial directions. The corneas were post-fixed in 1% aqueous osmium tetroxide (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) and coated with a gold alloy after dehydration. Corneal endothelial transition zone images were collected using high-resolution field-emission scanning electron microscopy (SEM) (SU 8010, Hitachi High Technologies Corp., Tokyo, Japan).

Immunostaining of the corneal endothelial transitional zone (flat mount): Fixation was performed for 30 min in methanol at room temperature. Whole flat-mounted corneal tissues were treated with a blocking solution in phosphate-buffered saline (PBS) containing 2% heat-inactivated goat serum (Cell Signaling Technology, Danvers, MA) and 2% bovine serum albumin (BSA; VWR International, Radnor, PA) for 1 h at room temperature.

We evaluated and compared the expression levels of p75NTR, SOX9 [9], Lgr5 [11], TERT [12], and K_i-67 in the transition zone between three laboratory animals. Immunostaining was performed using p75NTR antibody (Cell Signaling Technology, Danvers, MA), SOX9 antibody (Abcam, Cambridge, MA), Lgr5 antibody (Origene Technologies Inc., MD), TERT antibody (Thermo Fisher Scientific, MA), and K_i-67 antibody (Novus Biologicals, Centennial, CO) (Table 1). The sections were incubated with an antibody diluent solution (Invitrogen) at room temperature for 1 h and then incubated with the primary antibody (1:100) at 4 °C for 16 h. The following day, the sections were washed three times for 5 min with PBS in 0.1% Tween-20 and incubated with secondary antibodies (Table 2). After staining the sections with 4’,6’-diamidino-2-phenylindole (Thermo Fisher Scientific), images were obtained using a BX43 OLYMPUS fluorescence microscope (Olympus, Tokyo, Japan) and IS Capture software. Five random areas of the transition zone were selected, and the number of positive cells was counted for each area. For each assay, positive and negative controls were performed. The samples were exposed to both primary and secondary antibodies or were exposed only to the secondary antibody. A bright-field examination of the samples was subsequently performed to identify the proper antibodies and to check a false-positive staining reaction

RESULTS

Observation of flat-mounted corneas: On flat mounts, the endothelial cells in the transition zone had an irregular shape with slightly elongated nuclei compared with those of central endothelial cells, which had a hexagonal cell shape with round nuclei in all laboratory animals. Cell clusters with two or three layers were identified in this area. Proximity to the trabecular meshwork was associated with weaker ARS staining and a greater polymorphism of endothelial cells in the transition zone. The difference in the degree of staining is presumed to be due to differences in corneal thickness between laboratory animal species. (Figure 1).

Scanning electron microscopy examination findings: We observed the corneal endothelial transition zone using SEM

Table 1. Primary antibodies.

Antibody	Animal source	Clone	Source/ Catalog ID	Dilute concentration	
				Primary	Secondary
K_i-67	Mouse	8D5	Novus biologicals / NBP2–22112	1:100	1:500
p75NTR	Rabbit	D4B3	Cell signaling technology / #8238	1:100	1:500
SOX9	Rabbit	Polyclonal	Abcam / ab26414	1:100	1:500
Lgr5	Mouse	OTI2A2	Origene / TA503316	1:100	1:500
TERT	Mouse	2C4	Thermo fisher scientific / MA5–16034	1:100	1:1000

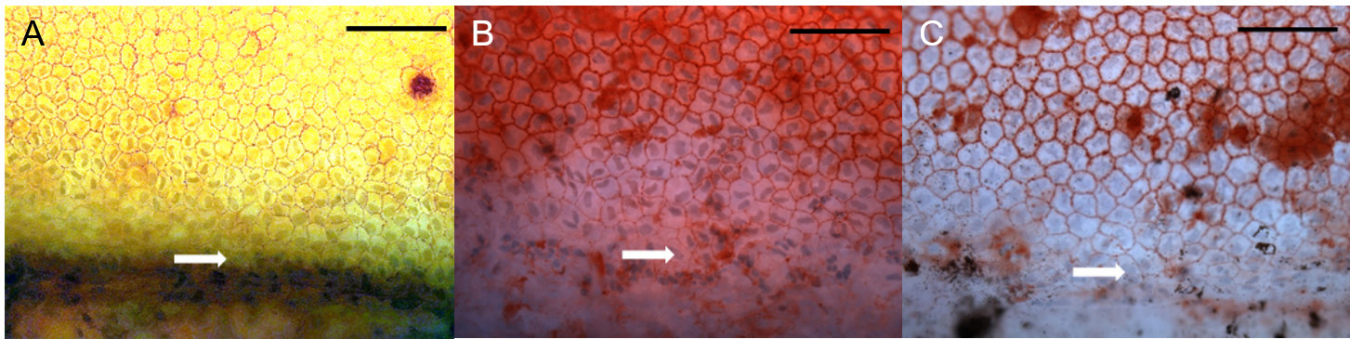

Figure 1. En face view of the corneal endothelial transition zone (white arrow) between the corneal endothelium and trabecular meshwork in different laboratory animals. Some cell clusters with two or three layers were identified; moreover, weaker Alizarin Red staining and greater polymorphic cells were observed in rabbit (A), rat (B), and mouse (C) specimens. The scale bar indicates 100 µm.

and found differences among the three laboratory animals (Figure 2). The adult rabbit corneas showed a lack of distinct and smooth transition zone structures, and the trabecular meshwork architecture transitioned immediately to the extreme peripheral endothelium, with relatively regularly arranged hexagonal endothelial cells in all directions. The high-power view revealed that the endothelial cells in the transition zone had an irregular shape with less dense, less wavy intercellular junctions compared to those associated with the central endothelial cells. In the murine corneas, a smooth transition zone was negligibly detected. Notably, in the transition zone of the rat and mouse corneas, a distinctive intercellular cystic space was identified, in contrast to that of the rabbit.

Immunostaining of the corneal endothelial transition zone and comparison of K_i-67 expression between three laboratory animals: The proliferative activity in the transition zone was evaluated using the K_i-67 marker, a nuclear protein associated with cell proliferation. In rabbit corneal flat mounts, the mean number of K_i-67 positive cells was 2.5 ± 1.9 in five random areas of the transition zone. In the murine corneas, extremely rare expression and the mean number of K_i-67 positive cells were 0.4 ± 0.5 (rat) and 0.8 ± 0.8 (mouse) in five random areas of the transition zone (Figure 3).

p75NTR—Fluorescent staining for p75NTR was observed in the transition zone in the rabbit cornea. The mean number of positive cells was 1.6 ± 1.3 in five random areas of the transition zone. Conversely, no reaction was observed in either murine cornea (Figure 4A).

Lgr5—Fluorescent staining for Lgr5 was also observed in the transition zone in the rabbit cornea. The mean number of positive cells was 1.8 ± 0.4 in five random areas of the transition zone. However, no reaction was observed in either murine cornea (Figure 4B).

SOX9—SOX9 activity was detected in the transition zone of the rabbit corneal flat mounts. The mean number of positive cells was 1.2 ± 0.4 in five random areas of the transition zone. However, no reaction was observed in either murine cornea (Figure 4C).

TERT—Immunofluorescence staining for TERT was observed in the transition zone of the rabbit corneal flat mounts. The mean number of positive cells was 1.4 ± 0.9 in five random areas of the transition zone. TERT was not expressed in the rat or mouse corneas (Figure 4D).

DISCUSSION

This comparative study explored the microstructures of the corneal endothelial transition zone between three different laboratory animals: rabbits, rats, and mice. Studies have reported on the microanatomy of the transition zone in the posterior limbus of the human cornea [1-4]; however, few studies have reported on those of nonhuman species [8,11,12].

In our study, endothelial cells in the transition zone had an irregular shape with slightly elongated nuclei and showed

Table 2. Secondary antibodies.

Antibody	Conjugation	Absorption/ Emission	Source/Catalog ID
Goat anti-rabbit IgG	Alexa fluor 488	495nm/519nm	Cell signaling technology/#4412S
Goat anti-mouse IgG	Alexa fluor 594	590nm/617nm	Cell signaling technology/#8890S
Goat anti-mouse IgM	Alexa fluor 488	495nm/519 nm	Thermo fisher scientific/A-21042

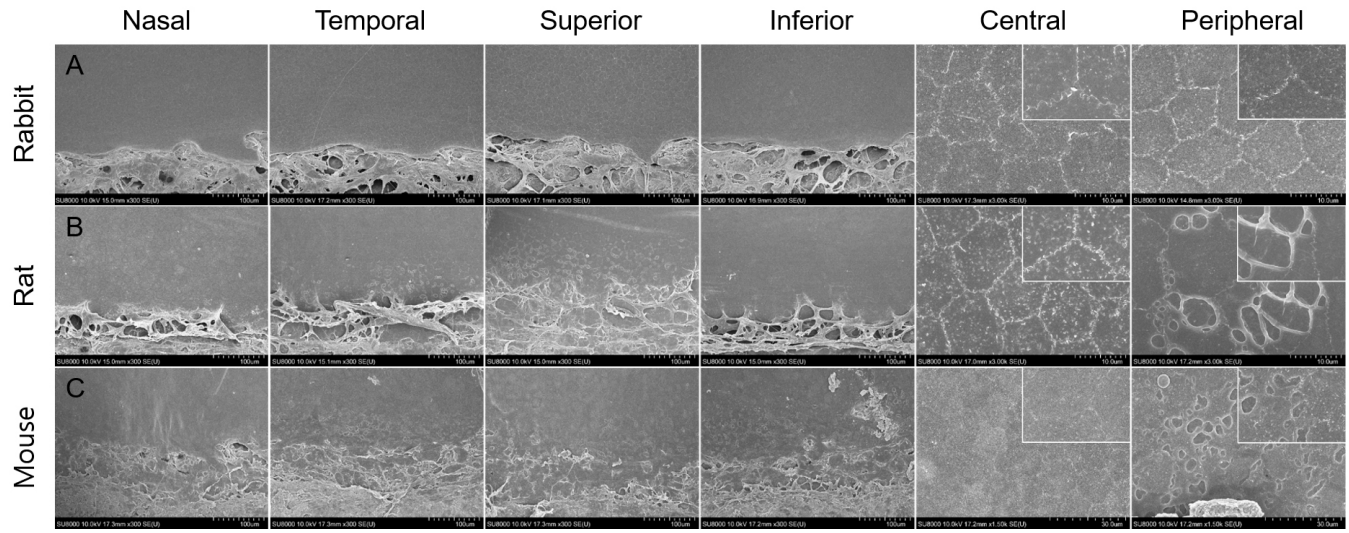

Figure 2. The corneal endothelial transition zone observed via scanning electron microscopy. The corneal endothelial transition zone in four directions and a high-power view of the central and transition zones in three laboratory animals.

overlapping and irregular arrangements with weaker ARS staining in all laboratory animals. These findings were similar to those observed in humans and may indicate weaker intercellular junctions and higher cell mobility than in the central cornea [2,8].

Using SEM, previous reports showed a well demarcated smooth transition zone in humans [2,8]; however, our animal corneas lacked distinct and smooth structures. Their trabecular meshwork architecture immediately transitioned to the extreme peripheral endothelium. These results differed somewhat from those of previous studies [8,12], which demonstrated that bovine and murine corneas had no apparent transition zones, whereas rabbit corneas had well demarcated zones. This may be explained by the individual or interspecies variation of this zone, as described in a previous report [8]. Nevertheless, the endothelial cells in this area also had irregular shapes, with less dense, less wavy intercellular junctions at high magnification compared to central endothelial cells. In particular, murine corneas exhibited unique intercellular spaces, and these SEM results may indicate weaker intercellular junctions and higher cell mobility in the transition zone.

Our flat preparation and ultrastructural findings of the endothelial transition zone suggested the existence of a proliferative capacity in this area in three laboratory animals. Therefore, we attempted to confirm the proliferative capacity (i.e., the presence of progenitor cells).

We first examined proliferative activity in the transition zones of three laboratory animals. K_i-67 has been widely used as a proliferation marker and is detected during the

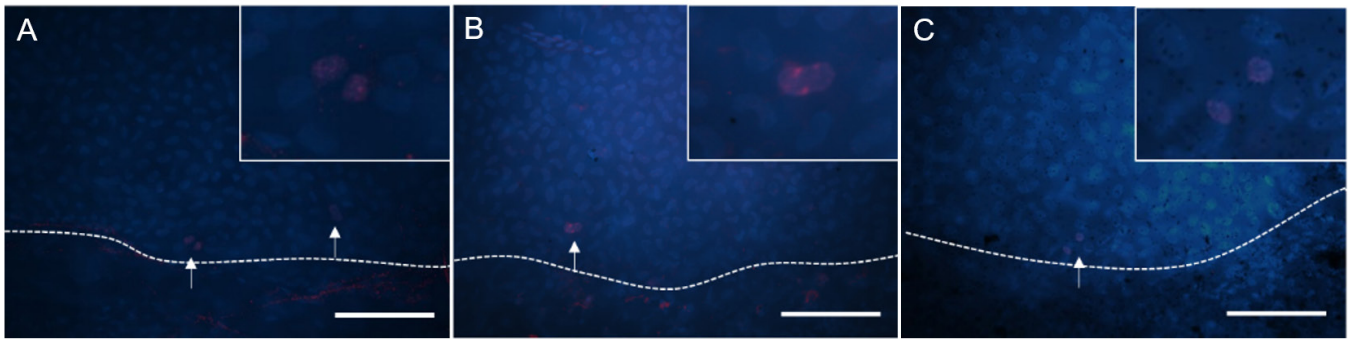

Figure 3. In the rabbit corneal flat mount, the mean number of K_i-67-positive cells was 2.5 ± 1.9 in five random areas of the transition zone (A). In murine corneas, rare expression and the mean number of K_i-67-positive cells were 0.4 ± 0.5 (B, rat) and 0.8 ± 0.8 (C, mouse) in five random areas of the transition zone. The scale bar indicates 100 µm.

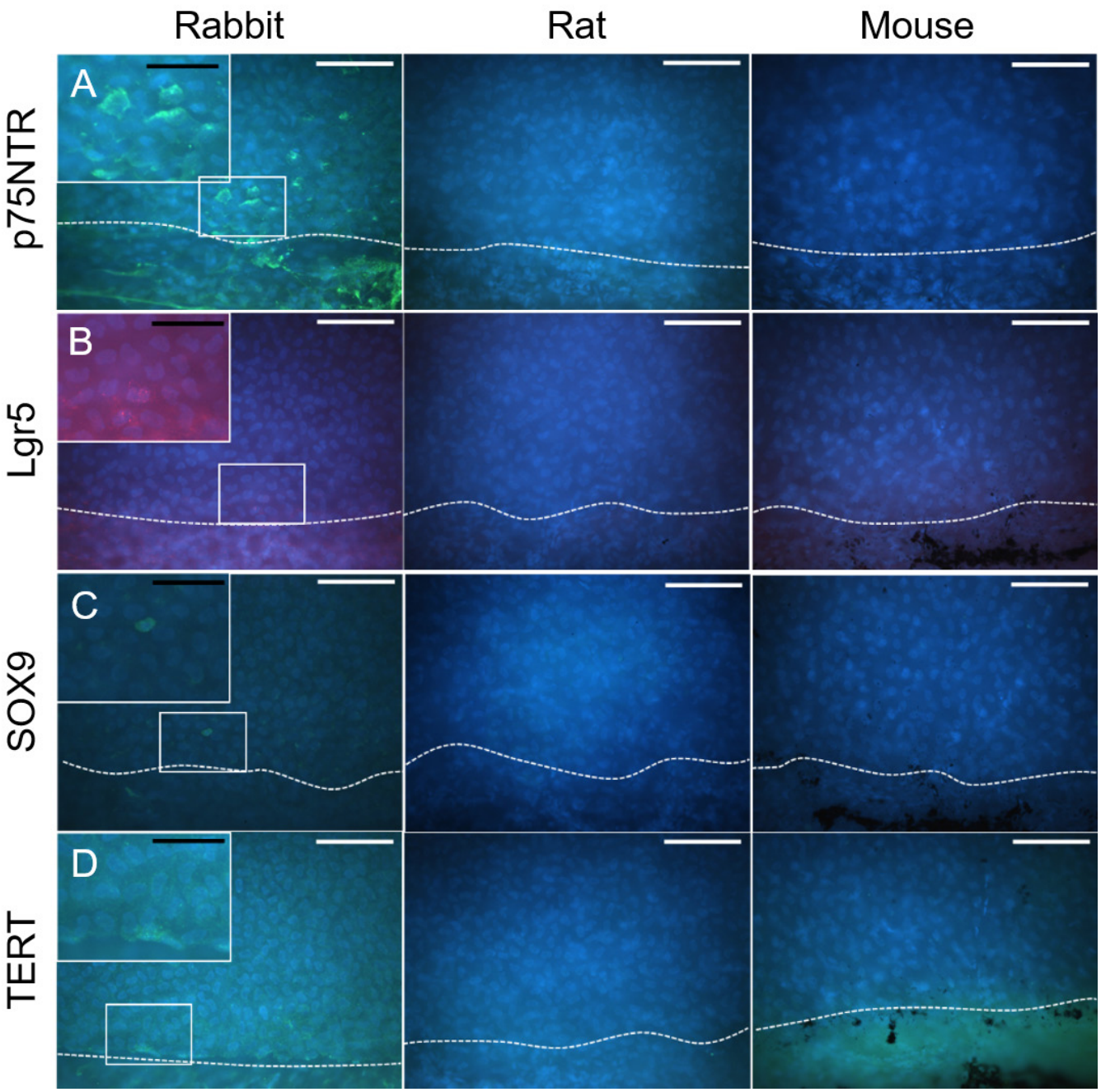

Figure 4. Fluorescent staining for the p75 neurotrophin receptor, leucine-rich repeat-containing G protein-coupled receptor 5, SRY-box transcription factor 9, and telomerase reverse transcriptase occurred in the transition zone in a rabbit cornea. No reaction was noted in either murine cornea. The white scale bar indicates 100 µm. The black scale bar indicates 50 µm.

active phases of the cell cycle [11,13,14]. We found proliferative activity in the transition zone of rabbits; however, murine corneas showed extremely rare expression in this zone. A previous report that analyzed K_i-67 expression and labeled cells with 5-bromo-2'-deoxyuridine in mouse corneal endothelium at different ages found that proliferation is active in mice at postnatal day three but not at postnatal day 30, and mature endothelium loses the ability to reproduce [11]. However, we confirmed that little proliferative capacity remained in the adult murine transition zone compared to the central endothelium without expression.

Previous studies have reported on human corneal endothelial progenitor cells [1,8-10,15-17]. Yam et al. detected the expression of Lgr5, TERT, and p75NTR by immunofluorescence analysis in the human endothelial transition zone [1]. SOX9- and p75NTR-positive cells were identified by immunohistochemistry in the human corneal endothelium [9]. According to these reports, progenitor cells are mainly present in the transition zone between the peripheral endothelium and the trabecular meshwork. However, in the animal transition zone, progenitor cells have been reported only in young mice and bovines [8,11,12]. We demonstrated the expression of four progenitor cell markers—p75NTR, SOX9, Lgr5, and TERT (i.e., neural crest and periocular mesenchymal markers)—in the rabbit corneal endothelial transition zone. Based on these data, we confirmed the presence of progenitor cells with proliferative capacity in the rabbit corneal endothelial transition zone. However, we could not confirm the presence of progenitor cells in the murine corneal endothelium. We performed positive and negative control staining to exclude possible differences in antibody reactivity between animal species. Because we observed that the control staining results for rabbits, rats, and mice did not differ, we thought that there was no difference in the cross-reactivity of antibodies between animal species.

Espana et al. evaluated the presence of corneal endothelial progenitor cells at different ages in mice [11]. In the corneal endothelium, the expression of four progenitor markers—SOX9, Lgr5, nestin, and NGFR—was observed in three-day-old mice. However, in 30-day-old mice, the expression of these four progenitor cell markers was absent or downregulated. We evaluated the expression of p75NTR, Lgr5, SOX9, and TERT in a 49-day-old mouse and rat and found no significant expression of these markers. Yoshida et al. observed low expression of cyclin-dependent kinase inhibitor 1B (p27kip1) and high expression of proliferating cell nuclear antigen (PCNA) in one-day-old mice, whereas in 11-day-old mice, high expression of p27kip1 was detected, and PCNA-positive cells were rare in the central and peripheral corneal endothelium with immunocytochemistry [18]. We used different animals aged seven weeks in our study. Unlike rabbits, the absence of significant expression of progenitor markers in the murine corneal endothelium is thought to be due to differences in corneal maturity, considering the lifespan. A limitation of our study is that we did not compare the differences in the corneal endothelial transition zone at different ages in each laboratory animal.

In conclusion, our study assessed the microstructure of the corneal endothelial transition zone and the presence of corneal endothelial progenitor cells in this zone in three laboratory animals: rabbits, rats, and mice. Although the transition zone was not identified clearly, unlike in human tissue, irregular cell morphology and loss of cell–cell contact were found in all laboratory animal corneal endothelial transition zones. The proliferative capacity and presence of progenitor cells were confirmed in the transition zone, especially in the rabbit cornea. These results may help develop treatments to stimulate corneal endothelial regeneration *in vivo* as an animal model.

ACKNOWLEDGMENTS

This research was supported by the Bio & Medical Technology Development Program of the NRF & funded by the Korean government (MSIT; No.2019M3E5D1A02069616) and by INHA UNIVERSITY HOSPITAL Research Grant. Conflicts of Interest: The authors have no financial conflicts of interest.

REFERENCES

1. Yam GH, Seah X, Yusoff NZBM, Setiawan M, Wahlig S, Htoon HM, Peh GSL, Kocaba V, Mehta JS. Characterization of human transition zone reveals a putative progenitor-enriched niche of corneal endothelium. Cells 2019; 8:1244- [PMID: 31614883].

2. Sub Koo O. Myung Kyoo Ko. Transitional zone of the corneal endothelium: flat preparation and ultrastructural findings. J Korean Ophthalmol Soc 2007; 48:691-8. .

3. Amann J, Holley GP, Lee SB, Edelhauser HF. Increased endothelial cell density in the paracentral and peripheral regions of the human cornea. Am J Ophthalmol 2003; 135:584-90. [PMID: 12719063].

4. He Z, Campolmi N, Gain P, Ha Thi BM, Dumollard JM, Duband S, Peoc'h M, Piselli S, Garraud O, Thuret G. Revisited microanatomy of the corneal endothelial periphery: new evidence for continuous centripetal migration of endothelial cells in humans. Stem Cells 2012; 30:2523-34. [PMID: 22949402].

5. Joyce NC. Proliferative capacity of corneal endothelial cells. Exp Eye Res 2012; 95:16-23. [PMID: 21906590].

6. Kinoshita S, Koizumi N, Ueno M, Okumura N, Imai K, Tanaka H, Yamamoto Y, Nakamura T, Inatomi T, Bush J, Toda M, Hagiya M, Yokota I, Teramukai S, Sotozono C, Hamuro J. Injection of cultured cells with a ROCK inhibitor for bullous keratopathy. N Engl J Med 2018; 378:995-1003. [PMID: 29539291].

7. Tuft SJ, Williams KA, Coster DJ. Endothelial repair in the rat cornea. Invest Ophthalmol Vis Sci 1986; 27:1199-204. [PMID: 3733367].

8. Sie NM, Yam GH, Soh YQ, Lovatt M, Dhaliwal D, Kocaba V, Mehta JS. Regenerative capacity of the corneal transition

zone for endothelial cell therapy. Stem Cell Res Ther 2020; 11:523-[PMID: 33276809].

9. Hara S, Hayashi R, Soma T, Kageyama T, Duncan T, Tsujikawa M, Nishida K. Identification and potential application of human corneal endothelial progenitor cells. Stem Cells Dev 2014; 23:2190-201. [PMID: 24588720].

10. McGowan SL, Edelhauser HF, Pfister RR, Whikehart DR. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. Mol Vis 2007; 13:1984-2000. [PMID: 17982423].

11. Espana EM, Sun M, Birk DE. Existence of corneal endothelial slow-cycling cells. Invest Ophthalmol Vis Sci 2015; 56:3827-37. [PMID: 26066751].

12. Yu WY, Grierson I, Sheridan C, Lo AC, Wong DS. Bovine posterior limbus: an evaluation of an alternative source for corneal endothelial and trabecular meshwork stem/progenitor cells. Stem Cells Dev 2015; 24:624-39. [PMID: 25323922].

13. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31:13-20. [PMID: 6339421].

14. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984; 133:1710-5. [PMID: 6206131].

15. Whikehart DR, Parikh CH, Vaughn AV, Mishler K, Edelhauser HF. Evidence suggesting the existence of stem cells for the human corneal endothelium. Mol Vis 2005; 11:816-24. [PMID: 16205623].

16. Katikireddy KR, Schmedt T, Price MO, Price FW, Jurkunas UV. Existence of neural crest-derived progenitor cells in normal and fuchs endothelial dystrophy corneal endothelium. Am J Pathol 2016; 186:2736-50. [PMID: 27639969].

17. Zhang J, Ahmad AM, Ng H, Shi J, McGhee CNJ, Patel DV. Successful culture of human transition zone cells. Clin Exp Ophthalmol 2020; 48:689-700. [PMID: 32249477].

18. Yoshida K, Kase S, Nakayama K, Nagahama H, Harada T, Ikeda H, Harada C, Imaki J, Ohgami K, Shiratori K, Ilieva IB, Ohno S, Nishi S, Nakayama KI. Involvement of p27KIP1 in the proliferation of the developing corneal endothelium. Invest Ophthalmol Vis Sci 2004; 45:2163-7. [PMID: 15223790].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 17 March 2024. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.