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In Vivo Labeling and Tracking of Proliferating Corneal Endothelial Cells by 5-Ethynyl-2′-Deoxyuridine in Rabbits

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Purpose: To develop a method to label proliferating corneal endothelial cells (ECs) in rabbits in vivo and track their migration over time.

Methods: We compared intraperitoneal (IP) and intracameral (IC) administration of 5-ethynyl-2′-deoxyuridine (EdU) in two experiments: (1) six rabbits received IP or IC EdU. Blood and aqueous humor (AH) samples were incubated with HL-60 cells. Flow cytometry detected the EdU incorporation, representing the bioavailability of EdU. (2) In vivo EdU labeling was investigated in pulse-chase study: 48 rabbits received EdU IP or IC. The corneas were flat-mounted after 1, 2, 5, or 40 days and imaged using fluorescence microscopy. EdU^+ and $Ki67^+$ ECs were quantified and their distance from the peripheral endothelial edge was measured.

Results: EdU was bioavailable in the AH up to 4 hours after IC injection. No EdU was detected in the blood or the AH after IP injection. High quality EdU labeling of EC was obtained only after IC injection, achieving 2047 ± 702 labeled ECs. Proliferating ECs were located exclusively in the periphery within 1458 ± 146 µm from the endothelial edge. After 40 days, 1490 ± 397 label-retaining ECs (LRCs) were detected, reaching 2219 ± 141 µm from the edge, indicating that LRCs migrated centripetally.

Conclusions: IC EdU injection enables the labeling and tracking of proliferating ECs. LRCs seem to be involved in endothelial homeostasis, yet it remains to be investigated whether they represent endothelial progenitor cells.

Translational Relevance: EdU labeling in animal models can aid the search for progenitor cells and the development of cell therapy for corneal endothelial dysfunction.

Introduction

Corneal endothelial dysfunctions are currently treated with corneal transplantation[1,2]; however, more than one-half of the world's population does not have access to this treatment and alternative solutions are urgently needed.[2] Options could be in vivo pharmacologic or surgical stimulation of endothelial cell (EC) proliferation or cell therapy using in vitro cultivated ECs obtained from mature or progenitor ECs.[3] However, the existence of EC progenitors remains to be demonstrated. Emerging clinical and preclinical evidence points toward a reserve of proliferative ECs and progenitor cells in the extreme periphery of the corneal endothelium: peripheral ECs have higher replicative capacity than central ECs[4,5] and express progenitor cell markers,[6–8] and in patients, spontaneous repopulation of a central denuded Descemet membrane has been described.[9,10] Pulse-chase studies, using ^{3}H-thymidine, 5-bromo-2′-deoxyuridine or 5-ethynyl-2′-deoxyuridine (EdU) have been used in

various tissues, but rarely in corneal endothelium, to label and track slow-cycling label-retaining cells, assumed to be progenitor cells.[11–13] In the present study, we aimed to develop a method to label proliferating ECs with EdU in rabbits in vivo. We compared intraperitoneal (IP) and intracameral (IC) injections of EdU with regard to the (1) bioavailability of EdU and (2) in vivo EdU labeling of ECs. The purpose was to localize and track label-retaining ECs (LRCs), which are possibly progenitor cells.

Methods

Animals and Ethics Statement

New Zealand White rabbits (KB Lidköpings Kaninfarm, Sweden) (5-week-old females, $n = 54$) were anesthetized by isoflurane gas inhalation and oxybuprocain eye drops. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal experiments were approved by the Danish national authority, called "The Animal Experiments Inspectorate."

Bioavailability of EdU in Blood and Aqueous Humor (AH)

EdU Administration

EdU (Thermo Fisher Scientific, Waltham, MA) was diluted in a balanced salt solution to 5 mg/mL (saturated solution). The rabbits were injected with EdU, either IP (10 mg/kg; $n = 3$) or IC, in both eyes through the cornea at the superotemporal limbus with a 30G needle (100 µL; $n = 3$).

Collection of Blood and AH Samples

Peripheral blood (ear vein) was collected 15 minutes and 4 hours after EdU injection and kept in a blood collection tube (BD Vacutainer plastic serum tube; BD, Franklin Lakes, NJ) at 5 °C for 30 minutes, before centrifugation at 2500 rpm at room temperature (RT) for 15 minutes. AH samples (approximately 300 µL) were collected through the limbus with a 27G needle after 15 minutes (right eye) and after 4 hours (left eye), and the animals were euthanized with intravenous pentobarbital. The samples were kept in Eppendorf tubes at −80 °C until further processing.

Cultivation of HL-60 Cell Cultures With Serum and AH Samples

HL-60 cells (ATCC; #CCL-240) were cultivated in RPMI1640 + $NaHCO_3$ in-house medium supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland), 100 µg/mL streptomycin, 100 U/mL penicillin, and 300 µg/mL L-glutamine (Thermo Fisher Scientific) and were maintained at 37 °C in 5% CO_2. The cells were seeded in a 96-well U-bottom plate (1×10^6 cells per well) and resuspended in 50 µL of serum or AH before incubation for 4 hours at 37 °C in 5% CO_2 with (1) blank serum and AH as negative controls, (2) sera and AH with known concentrations of EdU (10, 100, or 1000 µM) as positive controls, (3) sera and AH after IP EdU injection, and (4) sera and AH after IC EdU injection.

EdU-Staining Protocol and Flow Cytometry

The cells were fixed and stained for EdU using the Click-iT Plus EdU Alexa Flour 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific). In accordance with the manufacturer's protocol, the cells were washed once in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and fixed in Click-iT fixative for 15 minutes at RT, washed in 1% BSA in PBS, and resuspended in 1× Click-iT saponin-based permeabilization and wash reagent. The Click-iT reaction cocktail was added, and the cells were incubated for 30 minutes at RT. Subsequently, the cells were washed in 1X Click-iT saponin-based permeabilization and wash reagent. All cells in each well were analyzed with a Becton Dickinson LSR II flow cytometer using BD FACSDIVA software, version 8 (BD). Data were processed with FlowJo software, version 10.4.1 (BD).

In Vivo Labeling and Tracking of Proliferating ECs

EdU Administration

IP or IC EdU injection was done on day (D) 0, as described elsewhere in this article, and repeated after 24 and 48 hours to increase the number of labeled cells. Animals were euthanized after 28 hours (D1), 52 hours (D2), 5 days (D5), or 40 days (D40) after the initial injection (IP $n = 4$ and IC $n = 8$, at each time point) (see flow chart in Fig. 1). The D1 and D2 groups were euthanized 4 hours after the last EdU injection to allow time for the incorporation of EdU[14] and to optimize signal intensity.[15] The time points D1, D2, and D5 were chosen to study the effects of EdU administration on the EC proliferation. The chase period of 40 days was chosen to localize label-retaining cells and to track their migration. After euthanization, the 12 o'clock position of the eyeball was marked with a suture through the sclera. The eyes were enucleated immediately hereafter, and the corneoscleral buttons were dissected and fixed in 0.5% paraformaldehyde (pH 7.4) for 45 minutes.[16]

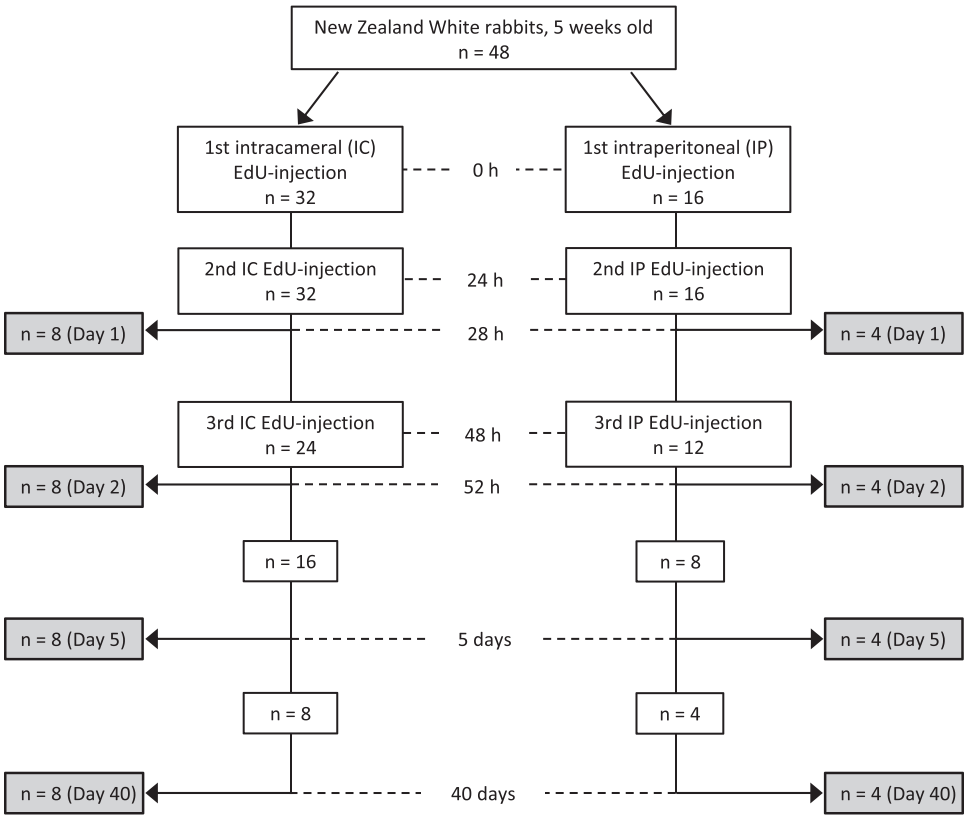

Figure 1. Flow chart of the in vivo EdU-labeling study (pulse-chase design). EdU was administered intracamerally or intraperitoneally in two or three pulses. Corneas were excised and fixed after different chase periods, resulting in eight final experimental groups as depicted in the gray boxes.

Ki67 and EdU Staining and Flat Mounting

EdU labeling was detected using the Click-iT Plus EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific), conforming to the manufacturer's protocol. After permeabilizing the cells with 1% Triton X-100 and blocking with 3% BSA in PBS, the endothelial side of the corneal buttons was covered with the EdU reaction cocktail and incubated for 30 minutes at RT. Subsequently, the corneas were incubated for 1 hour at 37 °C with Ki67 primary antibody (monoclonal mouse anti-human, MIB-1, unconjugated) (Dako, Glostrup, Denmark, M724029-2) diluted 1:200 in 3% BSA in PBS. The secondary antibody was goat anti-mouse conjugated with Alexa Fluor 555 (Thermo Fisher Scientific), diluted 1:500 in 3% BSA in PBS, and exposed to the endothelial side of the cornea for 45 minutes at 37 °C. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (5 µM in PBS; Nordic Biosite, Copenhagen, Denmark). Four radial incisions divided the superior, inferior, nasal, and temporal quadrants, and the corneas were flat mounted endothelial side up on a glass slide and covered with Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA) and a cover slip.

Microscopy

We used an LSM 710 on an Axio Imager Z2 upright microscope equipped with a Zeiss EC Plan-Neofluar ×10/0.3 objective and controlled with ZEN software (Carl Zeiss AB, Oberkochen, Germany) for confocal image acquisition. The complete endothelial surface was imaged manually and nonoverlapping images of the entire periphery 360°, from the transition zone and approximately 2.5 mm centripetally, were obtained by automatic mosaicking and Z-stacking with 10 µm steps.

Exhaustive Fluorescent Cell Count Method

The region of interest comprised the periphery from the endothelial edge and 2.3 mm centripetally. All $Ki67^+$ and EdU^+ cells (excluding the injection sites) were automatically counted in Fiji[17] using a macro with the following consecutive steps: filtering (Gaussian blur; sigma 1), maximum intensity projection, local thresholding (Phansalkar method[18]), watershed separation, and analyze particles (size, 30–500 µm^2; circularity, 0.6–1.0). Double-positive ECs ($Ki67^+$ and EdU^+) were counted automatically in Fiji by multiplying the mask images of the two channels,

followed by thresholding, segmenting, and counting the objects, as described elsewhere in this article. Ki67 is a cellular protein that is accumulated during the S, G2, and M-phases and is gradually degraded during G1 and G0 phases.[19] The Ki67$^+$ ECs represented proliferating ECs at the time of tissue fixation (D1, D2, D5, and D40). EdU labeling represented ECs in the S-phase during the EdU pulses (D0, D1, and D2), subtracting the loss of EdU incorporation owing to cell division or DNA repair.[20,21] The EC density was calculated as the mean of the peripheral and central 4′,6-diamidino-2-phenylindole nuclei counts in nonoverlapping 40× images of flat-mounted corneas ($n = 4$ in the center and periphery) using the CorneaJ plug-in for Fiji.[22] The total EC population was derived from the EC density and the endothelial surface area measured on flat-mounted corneas ($n = 4$ eyes at D1 and D40).

Localization of Proliferating and LRCs

Centripetal migration of ECs was assessed by measuring the distance from the endothelial peripheral edge to Ki67$^+$ and EdU$^+$ ECs at D1 and D40. The maximal distance per quadrant was calculated as the mean of three measurements in each quadrant.

Statistical Analysis

Groups were described by the mean ± standard deviation. A linear mixed effects model was used to analyze the effect of EdU administration route, quadrant, and time on the density of Ki67$^+$ cells (EC/mm^2). Likewise, the effect of quadrant and time on cell distance from the endothelial edge was analyzed for Ki67 and EdU separately. The random effect was the "eye," because correlation was expected between quadrants in the same eye. The model took in to account the repeated measurements, missing data, and variability between rabbits. A post hoc analysis was performed using the least squares (maximum likelihood) method with Bonferroni adjustment of *P*-values. Statistical tests were performed using SAS Enterprise Guide, version 7.11. The level of significance was set at a *P* value of less than 0.05.

Results

Bioavailability of EdU in Blood and AH

An HL-60 cell culture was incubated with serum and AH samples containing no EdU (negative controls) or known concentrations of EdU (positive controls). EdU was detected in all positive controls and in none of the negative controls (Fig. 2). Subsequently, an HL-60 cell culture was incubated with serum and AH collected 15 minutes or 4 hours after in vivo IP EdU injection or in vivo IC EdU injection. In vivo IP injection of EdU resulted in nondetectable levels of EdU in the HL-60 cell culture, regardless of incubation with either serum or AH (Figs. 2A, B). Likewise, in vivo IC injection of EdU resulted in nondetectable levels of EdU after incubation with serum, whereas EdU was detected in the cell culture after incubation with AH collected 15 minutes after the EdU injection and, to a lesser extent, when AH was collected after 4 hours (Figs. 2C, D).

In Vivo Labeling and Tracking of Proliferating ECs

Qualitative Assessment of Labeling After IC vs IP EdU Injections

EdU$^+$ ECs were localized exclusively in the corneal periphery. In vivo IC EdU injection resulted in strong EC fluorescence and low intereye and intraeye variation in fluorescence signal intensity. The EdU signal was strongest during D1 through D5, and the pixels of the digital microscopy image were saturated in most cells, indicating high EdU loading. The EdU signal was weaker at D40, as expected owing to cell division or DNA repair (Figs. 3A, B). Conversely, IP injection of EdU resulted in a suboptimal EdU fluorescence signal. In the 12 eyes at D1, D2, and D5, the signal was clearly visible only in 1 eye. In seven eyes, the signal-to-noise ratio was low and insufficient for automatic segmentation using Fiji, and in four eyes the signal was nondetectable. At D40 after IP injection, EdU was not visible (Figs. 3C, D).

Impact of IC vs IP EdU Injections on EC Proliferation

Ki67$^+$ ECs were present in all eyes and located exclusively in the corneal periphery, in a 1.5 mm wide zone in the corneal periphery, regardless of the EdU administration route. The Ki67$^+$ ECs were quantified to detect changes in baseline proliferation after EdU administration. For quantitative analysis, the inferior, medial, and lateral quadrants were pooled (inferior–medial–lateral), as were D1 and D2. At D1 and D2, the Ki67$^+$ ECs represented 0.31 ± 0.08% and 0.37 ± 0.14% of the entire corneal EC population in IC eyes and IP eyes, respectively ($P = 0.1646$). From D1 and D2 to D5, the proliferation remained stable in all quadrants in IP eyes ($P = 1.000$), whereas significant increases were seen in IC eyes: the density of Ki67$^+$ ECs in the superior quadrant increased from 35 ± 13 EC/mm^2 at D1 and D2 to 70 ± 23 EC/mm^2 at D5 ($P < 0.0001$), and in the inferior–medial–lateral quadrants

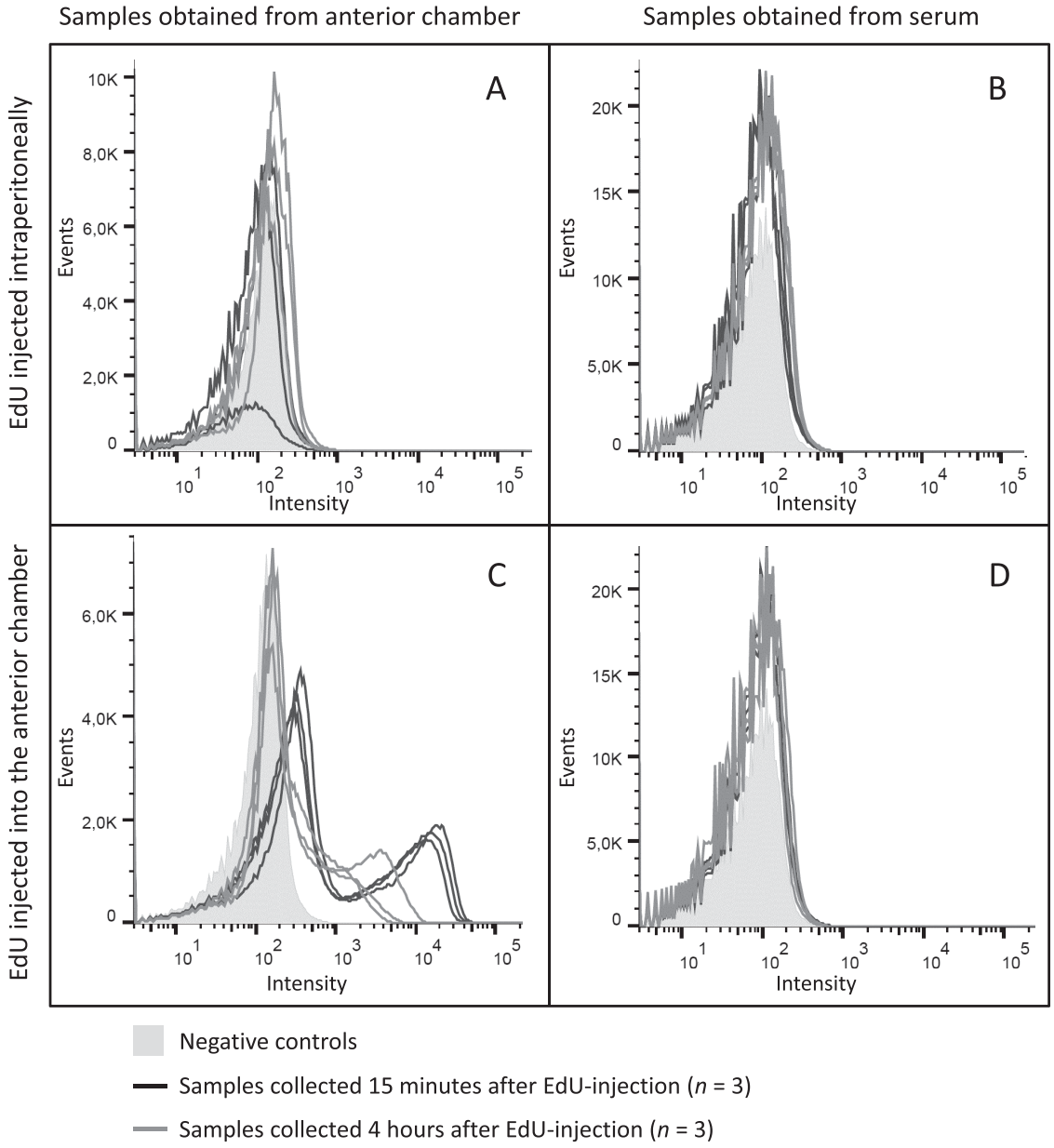

Figure 2. Histograms showing the incorporation of EdU into HL-60 cells incubated with AH (A, C) or sera (B, D) obtained from rabbits. When EdU was injected intraperitoneally, no EdU could be detected in either the AH or the serum (A–B). When EdU was injected into the anterior chamber of the eye, EdU was detectable after 15 minutes and after 4 hours in the AH (C), but not in the serum (D).

they increased from 26 ± 10 EC/mm^2 at D1 and D2 to 45 ± 16 EC/mm^2 at D5 ($P = 0.0002$).

Local Impact of the Repeated IC EdU Injections

At 4 hours after the injection, no excess proliferation was observed around the endothelio-Descemet rupture, whereas after 28 hours, an estimated 80% to 100% of the ECs at the lesion site (approximately 300 × 600 µm area) were Ki67$^+$. This decreased to an estimated 30% after 52 hours. After 72 hours, and at all time points hereafter, few, if any, ECs proliferated at the lesion site.

Quantification of EdU-Labeled Cells

EdU$^+$ ECs were counted only in IC eyes, and the injection sites were excluded from the analysis. At D2, representing maximum EdU loading, 2047 ± 702 ECs were EdU$^+$. At D40, we detected 1490 ± 397 LRCs, corresponding with 73 ± 19% of the EdU$^+$ ECs at D2. Occasionally, at D40, a few EdU$^+$ ECs were located further centrally than the 2.3 mm region of interest

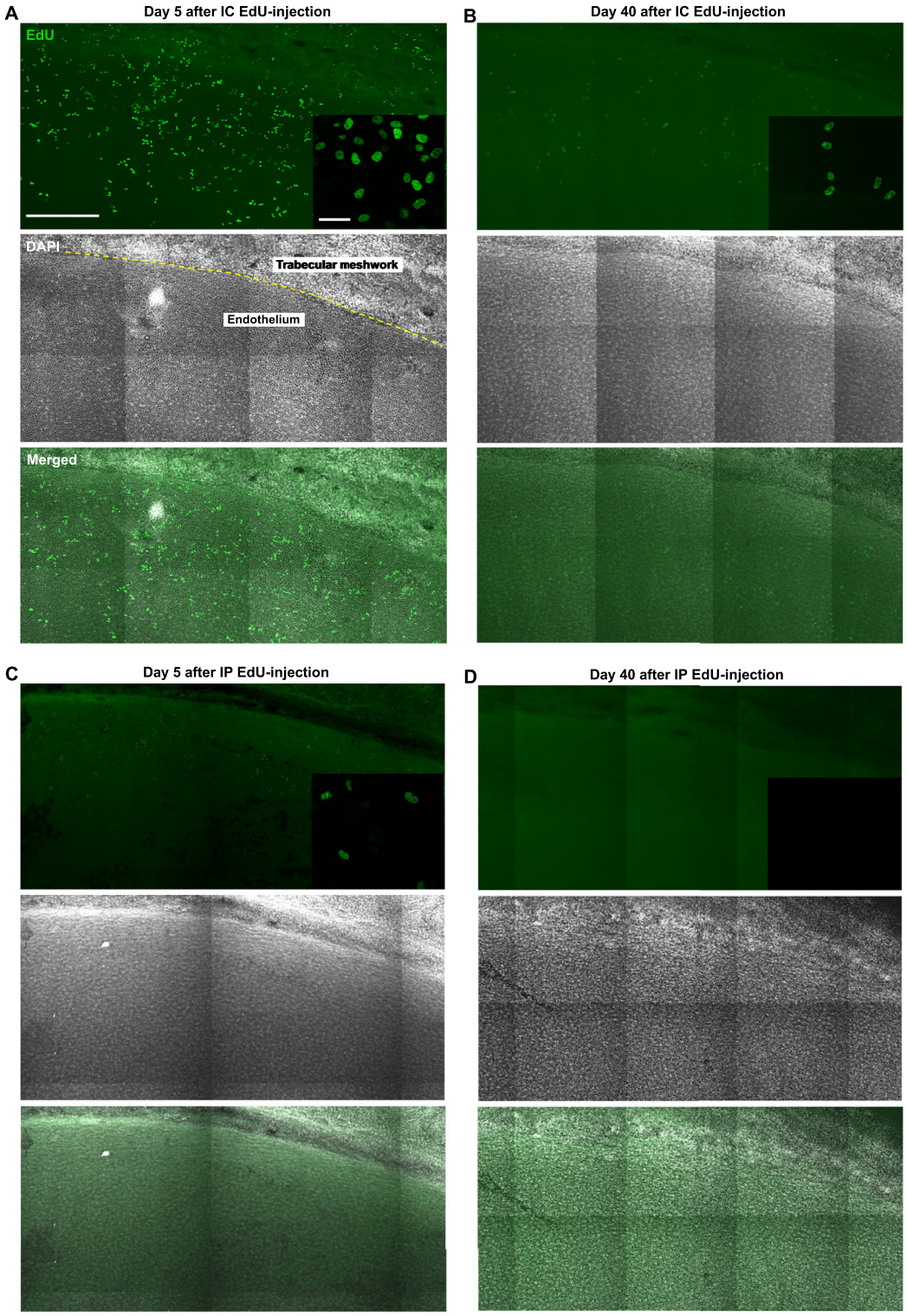

Figure 3. Mosaic images of flat-mounted corneas showing EdU$^+$ ECs (*green*) in the peripheral endothelium after rabbits were injected with EdU in vivo. EdU$^+$ ECs were located exclusively in the periphery, near the endothelial edge (*yellow dashed line*), and at the transition to the trabecular meshwork. No EdU$^+$ ECs were detected in the central cornea. IC injection of EdU resulted in a strong EdU-signal after 5 days (A), and a weaker, yet still detectable, signal after 40 days (B). IP injection of EdU resulted in a low EdU signal after 5 days (C) and no signal after 40 days (D). The maximum EdU loading was achieved at day 2, where 2047 ± 702 ECs were EdU$^+$. At day 40, a total of 1490 ± 397 label-retaining cells (LRCs) were detected, corresponding to 73 ± 19% of the EdU$^+$ ECs at day 2. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (*white*). *Scale bar*: 500 µm. Insets are magnified images of EdU$^+$ ECs (*green*), illustrating the EdU signal intensity, *scale bar:* 50 µm.

and were not counted. The majority of the EdU$^+$ ECs returned to a nonproliferating state within a few days: 81 ± 6% were Ki67$^-$ at D2, 98 ± 1% were Ki67$^-$ at D5, and 99.8 ± 0.3% were Ki67$^-$ at D40.

Centripetal Migration of EdU$^+$ ECs From the Periphery

At D1, there was no difference between the distribution areas of Ki67$^+$ and EdU$^+$ ECs in the corneal periphery ($P = 0.4964$). The width of the peripheral zone containing all Ki67$^+$ and EdU$^+$ ECs measured 692 ± 186 µm in the medial and lateral quadrants, 910 ± 200 µm in the inferior quadrant, and 1458 ± 146 µm in the superior quadrant ($P < 0.0002$). At D40, the width of the proliferative zone, hosting the Ki67$^+$ ECs, remained unaltered in all quadrants ($P > 0.1012$). In contrast, the EdU$^+$ ECs (LRCs) had moved centripetally at D40, reaching 619 ± 425 µm further centrally from the endothelial edge compared with D1 ($P < 0.0001$), corresponding with a migration speed of 16 µm/day. This finding was consistent in all quadrants, where the EdU$^+$ ECs at D40 in the medial, lateral, inferior, and superior quadrants reached 1306 ± 206 µm, 1129 ± 292 µm, 1499 ± 225 µm, and 2219 ± 141 µm, respectively, centripetally from the endothelial edge (Fig. 4). At D40, in five of eight corneas, a few EdU$^+$ cells were located further centrally than 2.3 mm, but only in the superior quadrant, reaching up to 2.5 mm. No Ki67$^+$ or EdU$^+$ ECs were observed in the center at any time point. It should be noted that from D1 to D40, the cornea white-to-white diameter, which was measured on flat-mounted corneas, increased from 1.2 ± 0.02 cm to 1.4 ± 0.02 cm ($P < 0.0001$).

Localization and Organization of Ki67$^+$ and EdU$^+$ ECs

An analysis of the combined Ki67 and EdU fluorescence revealed distinct patterns of cell organization, which could relate to their premitotic or

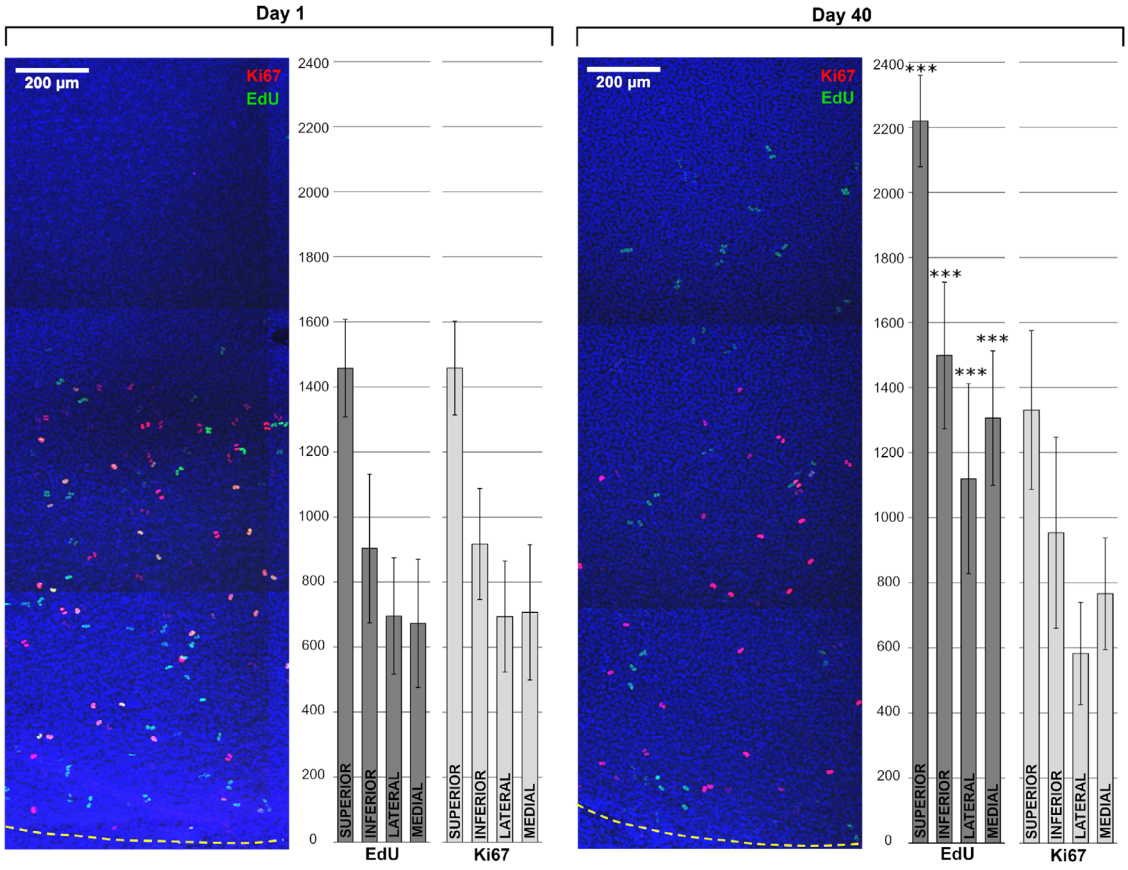

Figure 4. The localization of Ki67$^+$ (*red*) ECs and EdU-labeled ECs (*green*) is shown on flat-mounted corneas using confocal fluorescence microscopy. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). Bar graphs represent the maximum distance (microns) of Ki67$^+$ and EdU$^+$ ECs from the peripheral limit of the endothelium. Data are given as the mean ± standard deviation of three measurements in each quadrant. Proliferating ECs (Ki67$^+$) were present in all eyes and located exclusively in the periphery within a 1458 ± 146 µm wide zone starting at the endothelial edge (*yellow dashed line*). ECs were labeled with EdU, and after a chase period of 40 days the LRCs reached up to 2219 ± 141 µm from the edge (***$P < 0.0001$, compared with day 1), corresponding to a centripetal migration speed of 16 µm/day.

postmitotic status. The $Ki67^+/EdU^-$ ECs were arranged in a balanced combination of single cells and pairs of cells at all time points. The $Ki67^-/EdU^+$ ECs were almost exclusively in pairs at D1, D2, and D5, indicating that the cells had returned to the quiescent state after a division. The $Ki67^+/EdU^+$ (double positive) ECs were solely observed as single cells at all time points. At D40, the $Ki67^-/EdU^+$ LRCs were observed both in pairs and as single cells, but with a clear difference in their preferential location: the pairs were most often located further from the endothelial edge, toward the center, whereas the single cells were most often located closer to the peripheral edge of the endothelium. The few double positive ECs at D40 were mainly located close to the endothelial edge among the general population of $Ki67^+$ ECs and did not seem to be different from those cells.

Discussion

In this study, we successfully labeled corneal ECs with EdU using IC EdU injections. Labeling of DNA with pyrimidine nucleosides, constituting DNA synthesis markers,[23,24] has been widely used in rats and mice, with IP or subcutaneous injection as standard methods.[13,25] EdU has an advantage over ^{3}H-thymidine, 5-bromo-2′-deoxyuridine and ^{3}H-thymidine by being detected by click-it chemistry, enabling faster and gentler laboratory processing. IP injection of EdU is a simple procedure, but in rabbits, compared with mice and rats, the distribution volume is high. Accordingly, we find a low bioavailability of EdU in the AH after IP injection, resulting in nonreproducible, low-intensity labeling of ECs in vivo. In contrast, IC injection of EdU has the advantage of directly exposing the ECs to EdU.[26] We demonstrate that IC injections of EdU ensure bioavailability for at least 4 hours and successfully label ECs in vivo.

EdU is a potential confounder in studies with proliferation as an outcome, because EdU incorporation can halt the cell cycle. In this study, the entire peripheral endothelium constituted a proliferative zone, 1.5 mm wide, hosting $Ki67^+$ ECs. Locally at the injection site, the EC proliferation increased transiently owing to lifting of the cell–cell contact inhibition.[25] Universally in the peripheral zone, Ki67 was upregulated after IC, but not IP, EdU injections. This general upregulation can have two explanations: (1) a response to the repeated endothelio-Descemetic ruptures and (2) a compensation for the toxicity of integrated EdU.[20,21] Both explanations suggest intercellular communication regulating the peripheral proliferation. Hence, using IC EdU injections in an in vivo setting requires appropriate control groups to account for the potential confounding effect on baseline proliferation.

In a pulse-chase design, the cells that remain EdU^+ after a chase period are designated LRCs and are interpreted as slow-cycling, which is a feature of stem/progenitor cells.[27] During the chase, the number of EdU^+ ECs can be modulated by several mutually nonexclusive mechanisms: a few cell cycles increase the number of EdU^+ cells, multiple cell divisions dilute the EdU, and EdU can be removed owing to cell death or DNA repair. Finally, the cell cycle could be halted after incorporating EdU, keeping the EdU^+ cell number stable.[20,21] In our study, LRCs were abundantly present at D40 in the corneal periphery after IC EdU injection. The LRCs at D40 exhibited normal endothelial nuclear morphology and were stained weaker compared with earlier time points. This finding indicates that cells remained vital and removed EdU slowly through DNA repair processes or diluted EdU by mitosis. The presence of double positive LRCs, although rare, indicated that these ECs divided slowly, either having incorporated EdU 40 days earlier or being the descendant of such a cell. Our study shows that IC EdU injections can be applied in a pulse-chase study design with a chase period of up to 40 days.

In human adult corneas, our team previously identified microanatomical structures, indicating a slow centripetal migration of ECs throughout life.[7] We show, to our knowledge, for the first time in this well-studied animal model, indirect signs of centripetal migration of ECs: after 40 days, several ECs had migrated approximately 600 µm centripetally from their initial location; thus, the estimated migration speed was 16 µm per day. In addition, maturation-related growth of the cornea from the proliferative zone, shifting the entire EC monolayer centripetally, could also contribute to the change in location. However, not all LRCs were shifted toward the center at D40, and those that remained near the endothelial edge were primarily arranged as single cells. In contrast, the more centrally located LRCs at D40 were almost exclusively arranged in pairs. The reason for this arrangement is unknown, yet we speculate whether single LRCs in the periphery could be progenitor cells undergoing asymmetric mitosis (Fig. 5). A similar model exists for the corneal epithelium.[28,29] Nevertheless, further studies are needed to confirm asymmetric cell division. In addition, it remains to be investigated whether these cells truly contribute to the homeostasis of the monolayer or if they are only a vestige of the embryonic development.

Our study has some limitations. The EdU exposure was not continuous, but was in pulses of 4-hour

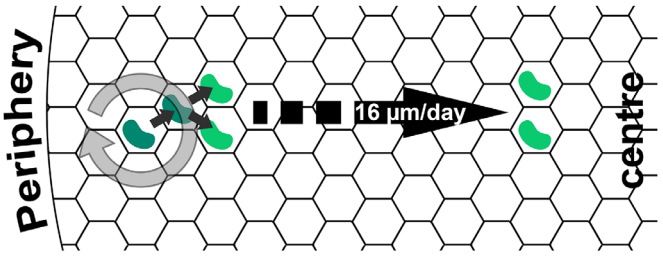

Figure 5. Model of corneal endothelial homeostasis. The rare endothelial progenitors located exclusively at the periphery of the cornea are suggested to be slow-cycling cells that divide asymmetrically. One of the daughter cells divides again, and the new cells slowly migrate to the center of the cornea.

durations, repeated with 24-hour intervals. Cells in the S-phase between the pulses may not have been labeled, leading to an underestimation of LRCs. EdU can impair cell cycle progression, particularly at high concentrations and long exposures.[21] In our in vivo study design, the exact concentration and exposure time could not be established, and the extent of EdU impairment was uncertain. An analysis of cell cycle dynamics would require double or triple staining with different nucleosides or cell cycle markers, such as cyclin and cyclin-dependent kinases.[15,30] The EdU toxicity could be further explored using cell death markers, e.g. the TUNEL assay or caspase activation. EdU molecules modified to minimize DNA damage[15,31] should be preferred in future studies of ECs.

Young rabbits comprise a suitable model to study corneal endothelial proliferation and regeneration owing to the highly proliferative endothelium. The young age of the rabbits in this study ensured a high number of EdU labeled ECs. In older animals, the proliferating ECs would be too few in number to report on the effectiveness of the labeling method. Results obtained in a rabbit model cannot be directly extrapolated to humans owing to interspecies variation. Nevertheless, it is reasonable to assume that many features are shared across species, e.g., the existence of endothelial progenitor cells.

In perspective, the IC EdU labeling technique we demonstrate in this study can be used as a tool to objectively measure the effect of EC therapy in animal models, for example, cell injection into the anterior chamber of the eye and drugs aimed at stimulating EC proliferation. For translation to humans, the pulse-chase study design can be applied in an ex vivo setting using an active storage machine allowing corneas to be preserved long term with viable ECs.[32] The identification of endothelial progenitor cells or ECs with high replicative competence would be crucial for the development of cell therapy for endothelial dysfunction.

In conclusion, this study shows that IC injection of EdU is a viable method to label and track proliferating ECs in rabbits in vivo. To our knowledge, IC injection of EdU has not been described previously in the literature. We demonstrate that LRCs reside in the periphery and we find indirect signs of centripetal EC migration. Further investigation is needed to determine whether the LRCs are progenitor cells.

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