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SURGICAL RETINAL EXPLANTS AS A SOURCE OF RETINAL PROGENITOR CELLS

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Purpose: To describe the novel observation of spontaneously migrating retinal cells from living donor surgical retinal explants that express progenitor cell markers in the absence of exogenous growth factors.

Methods: Surgical retinal explants were harvested from 5 consecutive patients undergoing 23 G pars plana vitrectomy for the management of rhegmatogenous detachment. During surgery, equatorial flap tears were trimmed with the vitreous cutter and aspirated. Excised tissue was then regurgitated into a syringe containing balanced salt solution and immediately transferred to tissue culture. Migrating cells subsequently underwent immunohistochemical staining and their characteristics were compared with those of a spontaneously immortalized Müller stem cell line.

Results: Spontaneously migrating cells were observed from samples taken from all 5 patients from Day 2 to 10 after transfer to culture. These cells were found to express embryonic cell markers, including paired box 6 (Pax6), sex-determining region Y-box 2 (Sox-2), nestin, cone-rod homeobox, and cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) as well as proteins consistent with early or retained differentiation down the Müller cell lineage, including glial fibrillary acidic protein and glutamine synthetase.

Conclusion: After injury, the human equatorial retina is capable of spontaneously producing cells that demonstrate migration and that express progenitor cell markers. In addition, these cells express proteins consistent with Müller cell lineage. These initial observations support the assertion that the human retina may possess the potential for regeneration and that surgical retinal explants could also act as a ready source of retinal progenitor cells.

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There is sustained and growing interest in the use of stem cells as a possible treatment for retinal dystrophy and degeneration,[1] which collectively account for the most prevalent causes of blindness registration in people of working and retirement age, respectively.[2] The mammalian neural retina demonstrates a limited ability to regenerate. However, it has been demonstrated that the Müller cells are capable of regenerating neural retina in multiple species, including fish,[3] chicken,[4,5] and rat.[6] Similarly, it has been demonstrated in several species that Müller cells can reenter the cell cycle (as indicated by the expression of progenitor markers) after acute injury, in turn suggesting differentiation into neural precursors before regeneration.[3,7,8] Furthermore, Müller cells and retinal neurons share common multipotent progenitor cells that persist in the mammalian retina in the late stages of development.[9] Isolation of Müller cells from peripheral cadaveric human retina and subsequent exposure to appropriate growth factors has demonstrated that these cells have the potential to transform into cells that possess the characteristics of multipotent stem cells.[10,11] Their potential for forming such cells seems to be related to retinal eccentricity, with the greatest potential for producing stem cells being demonstrated at the ora serrata in the so-called nonlaminated region of the retina.[12] Studies undertaken on pathological whole eye specimens or from vitreous samples during vitrectomy have been taken as evidence that cells of the ciliary epithelium proliferate within the vitreous base in the context of proliferative vitreoretinopathy.[13] Although such cells were initially believed to be "stem

cells," the evidence suggests that they maintain their initial differentiation (i.e., pigmented epithelium).[14]

In this article, we describe for the first time the spontaneous formation of cells that migrate from human equatorial retinal explants gathered during vitrectomy surgery for the emergency repair of retinal detachment. Surgical explants were stored after collection in standard tissue culture conditions without the addition of exogenous growth factors. These migrating cells express markers that suggest they reenter the cell cycle. The coexpression of Müller cell markers leads us to hypothesize that they are derived from human equatorial Müller cells. Such retinal explants are likely to be especially attractive to clinician-scientists; they are plentiful in busy retinal units, usually go to waste, but may offer a viable means of culturing human retinal progenitor cells.

Experimental Procedure

This study received approval from the local research ethics committee (HREC/17/POWH/537), and informed preoperative consent was obtained from five consecutive patients undergoing surgery for retinal detachment repair. During surgery, causative retinal tears were excised by a single surgeon (M.P.S.) with a 23-gauge (23 g) vitrectomy cutter (Constellation Vision System, Alcon, Fort Worth, TX) set at low cut rates (500–1,000 cuts/minute) using low vacuum. In all of the cases included in this study, the tears were located at, or slightly anterior to, the equator of the eye (i.e., at a significant distance posterior to the ora serrata). Each excised fragment was aspirated with the 23 g handpiece, and several retinal fragments measuring up to ≅0.42 mm^2 were obtained per patient (Table 1 and see **Video**, **Supplemental Digital Content 1**, http://links.lww.com/IAE/B421). Once the retinal tear was trimmed, the vitrectomy cutter was then externalized, and its tip is introduced into the opening of a 1-mL syringe preprimed with balanced salt solution (≅0.2 mL) and its contents were refluxed into the syringe. The tissue was then transferred immediately into a tissue culture insert containing 300 μL of prewarmed neurobasal A media supplemented with 2% B27 supplement, 1% N2 supplement, 0.8 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The culture insert was subsequently placed within a well of a 24-well cell culture plate containing 400 μL of supplemented neurobasal A media. The culture plate was then placed in an incubator with 5% CO_2 under controlled hypothermic conditions. Explants were maintained within the same wells; no tissue or cells were extracted for "passaging." The media in the culture insert was topped up and half of the media in the bottom well refreshed every alternate day.[15]

After 12 days of culture, migrating cells adherent on culture membranes (Figure 1) were fixed in 4% paraformaldehyde for 30 minutes. Each membrane was washed thrice with phosphate-buffered saline for 10 minutes. Fixed cells were stored in phosphate-buffered saline at 4°C until immunohistochemical staining. For immunofluorescent staining (Figures 2–4), cells were pretreated with 10% normal donkey serum in phosphate-buffered saline for 1 hour before overnight incubation at 4°C with primary antibodies: paired box 6 (Pax6) (Rabbit, 1:100; 901301 BioLegend, San Diego, CA), sex-determining region Y-box 2 (Sox-2) (Rabbit, 1:250, AB5603, EMD Millipore, Burlington, MA), cone-rod homeobox (CRX) (Mouse, 1:100, H00001406-M02; Novus Biologicals, Littleton, CO), nestin (Mouse, 1:100, MAB5326, EMD Millipore), P27 ^{Kip1} (Rabbit, 1:25, sc-528; Santa Cruz, TX), glial fibrillary acidic protein (GFAP) (Mouse, 1:100, MAB360, EMD Millipore), and glutamine synthetase (GS) (Mouse, 1:100, 610517; BD Biosciences, San Jose, CA) diluted in 1% serum and 1% Triton X-100 in phosphate-buffered saline. The cells were subsequently

Table 1. Donor Characteristics

Donor ID	Age (yr)	Sex	Duration of Symptoms	No. of Cultured Retinal Fragments	Average Fragment Size (mm^2)
1	53	M	4 days	3	0.42
2	65	M	7 days	5	0.39
3	46	F	7 days	1	0.29
4	65	M	3 weeks	5	0.24
5	56	M	5 days	2	0.25

M, male; F, female; duration of symptoms, days between onset of photopsia, floaters, or scotoma and surgery.

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incubated with species-specific secondary antibodies conjugated with Invitrogen Alexa Fluor 488 or 594 (Thermo Fisher Scientific, NSW, Australia) at 1:1,000 dilution for 4 hours at room temperature protected from light, followed by nuclear staining with Hoechst 33342 solution. Immortalized human Müller cell line (Moorfields/Institute of Ophthalmology) cells were used as a positive control for immunostaining (Figure 3). Negative

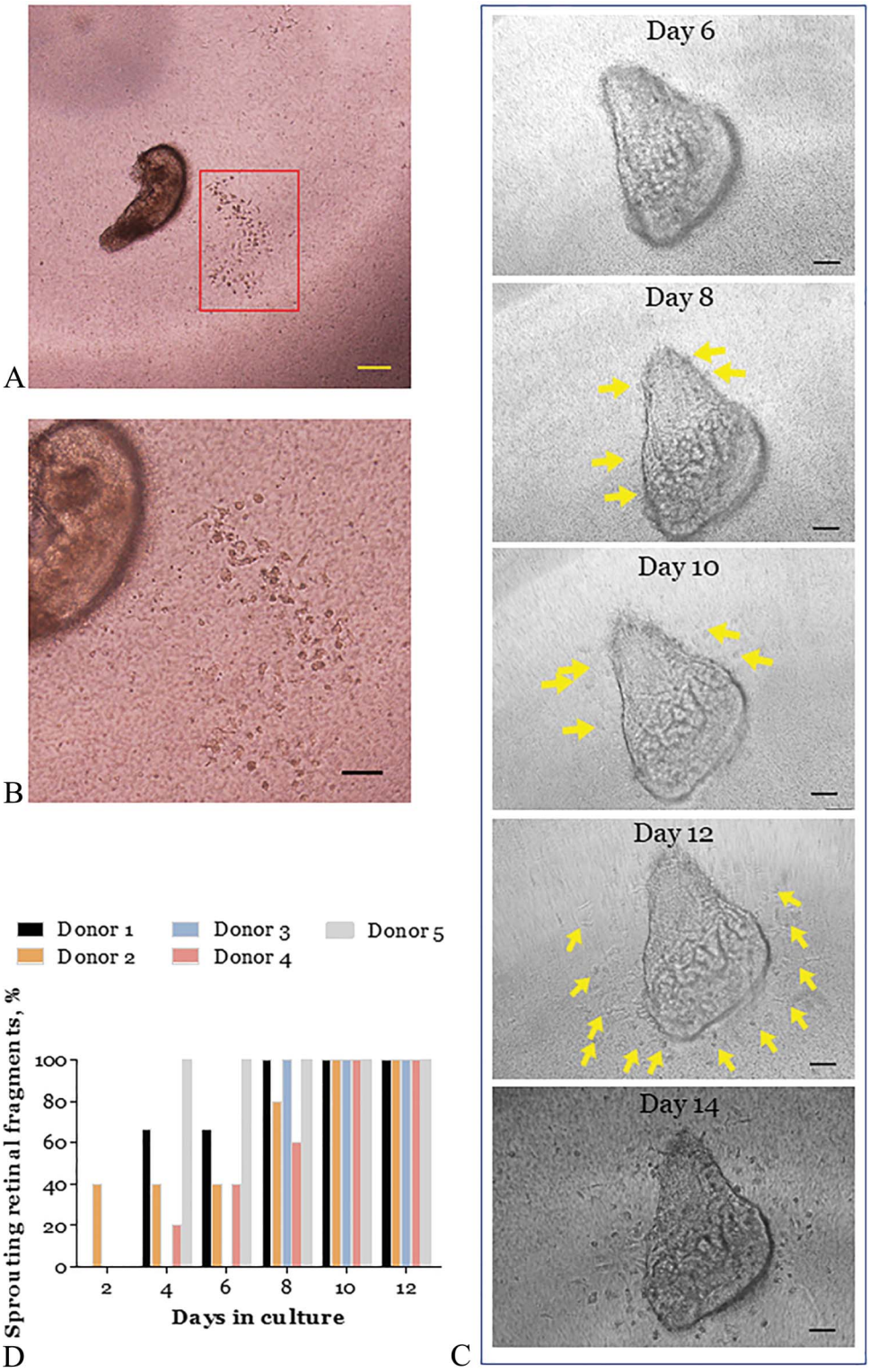

Fig. 1. Microscopic view of cells migrating from retinal explants collected from living donors during vitrectomy for rhegmatogenous retinal detachment. **A.** Representative image of cells migrating from an adjacent retinal explant collected from donor 1 after 4 days of culture. Red square indicates second population of migrating cells that are further away from retinal explant. **B.** Magnified view of migrating cells in the inset of (**A**). **C.** Microscopic images demonstrating progressive migration of cells from Day 6 to Day 14. Yellow arrow indicates migrating cells. **D.** Percentage of retinal fragments that generate migrating cells over 12 days of culture. Yellow bar: 200 μm; Black bar: 100 μm.

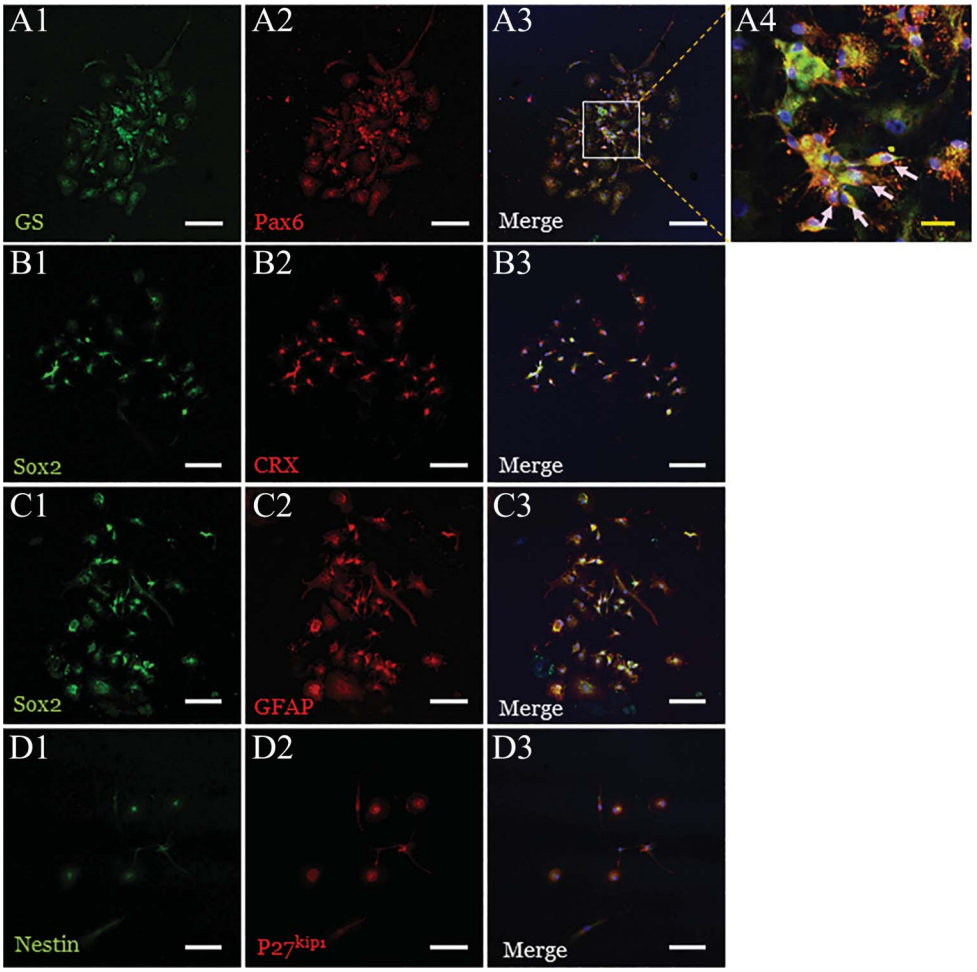

Fig. 2. Immunofluorescent examination of migrating cells. **A1–A4.** representative fluorescent images of migrating cells that are immunopositive for (**A1**) GS and (**A2**) Pax6. **A4.** Magnified view of inset in **A3**. Pink arrows point to a cell cluster from where individual cells migrated. **B1–B3.** Representative fluorescent images of migrating cells that are immunopositive for (**B1**) Sox-2 and (**B2**) CRX. **C1–C3.** Representative fluorescent images of migrating cells that are immunopositive for (**C1**) Sox-2 and (**C2**) GFAP. **D1–D3** Representative fluorescent images of migrating cells that are immunopositive for (**D1**) nestin and (**D2**) $p27^{kip1}$. White bar: 100 μm; yellow bar: 20 μm.Pax6 = paired box 6; Sox-2 = sex-determining region Y-box 2.

controls include staining without primary antibody or with isotype controls: rabbit polyclonal immunoglobulin G isotype (31235; Thermo Fisher Scientific), mouse IgG1 kappa (14-4714-81; Thermo Fisher Scientific), and mouse IgG2a kappa (14-4714-81; Thermo Fisher Scientific) (Figure 4). Immunofluorescent labeling of cellular markers was imaged on a LSM700 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) equipped with 405, 488, and 555 nm excitation lasers using Zeiss Efficient Navigation Black software.

Results

Retinal fragments from 5 adult patients aged from 53 to 65 years were collected during surgery for the repair of rhegmatogenous detachment (none with active proliferative vitreoretinopathy; see **Video**, **Supplemental Digital Content 1**, http://links.lww.com/IAE/B421). Spontaneously migrating cells were observed from Day 2 after transfer to tissue culture in the absence of growth factors; by Day 12, all retinal fragments had migrating cells (Figure 1). These migrating cells expressed embryonic cell markers, including Pax6 (retinal progenitor cell marker; upregulated after injury in the mammalian retina),[16] Sox-2 (multipotent stem cell marker; Sox-2 positive human Müller cells in culture have been demonstrated to possess stem cell characteristics),[10] CRX (photoreceptor progenitor cell marker), nestin (neuronal and glial progenitor marker protein),[17,18] and $p27^{kip1}$ (cell cycle regulator)[19,20] as well as proteins consistent with differentiation down the Müller cell lineage, including GFAP and GS (Figure 2). The overall immunohistochemical staining profile was similar to that of the positive control cells: the previously reported spontaneously immortalized Moorfields/Institute of Ophthalmology Müller cell line, which was obtained from cadaveric retina[11] and which is proposed to possess stem cell characteristics.[10]

Discussion

To the best of our knowledge, this is the first report of retinal cells expressing multiple progenitor cell

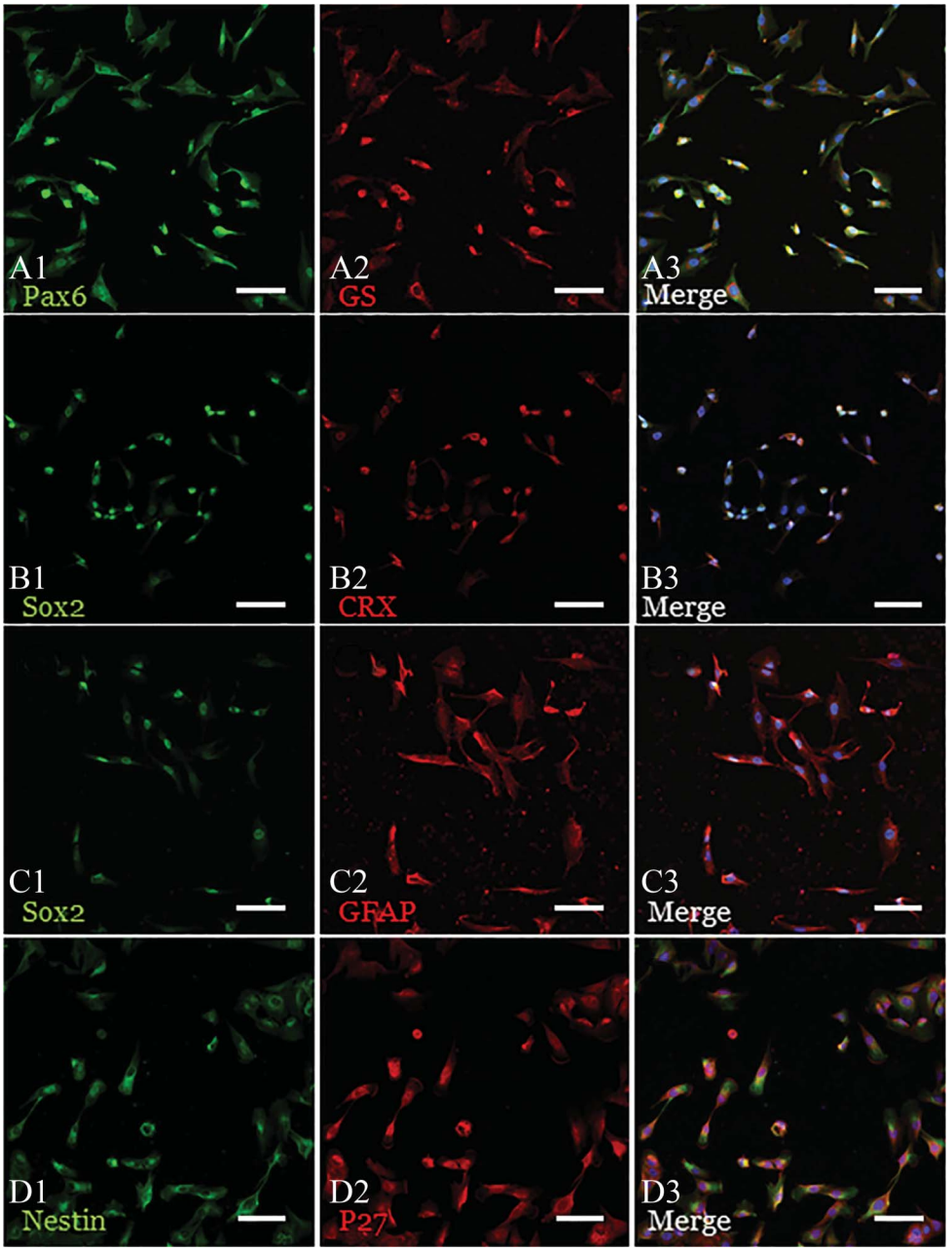

Fig. 3. Positive controls for immunofluorescent staining. Immunofluorescent staining of (**A1**–**A3**) GS and Pax6, (**B1**–**B3**) Sox-2 and CRX, (**C1**–**C3**) Sox-2 and GFAP, and (**D1**–**D3**) nestin and $p27^{kip1}$ in MIO cells. MIO, Moorfields/Institute of Ophthalmology.

markers derived from the retinal tissue of living human donors. These cells spontaneously migrated from equatorial/periequatorial retinal tissue fragments harvested during vitrectomy surgery for the primary repair of retinal detachment. Although previous reports suggest that the Müller cells may transform into progenitor cells after explantation in cadaveric studies (taken from a similar topographical location as our explants),[10] such cell lines were produced with the aid of exogenous growth factors[10,11]; this is in contrast to our findings, where we clearly demonstrate the migration of progenitor cells without the application of such factors. As noted above, migrating cells express embryonic cell markers including Pax6, Sox-2, CRX, nestin, and $p27^{kip1}$ and co-express proteins consistent with differentiation down the Müller cell lineage, including GFAP and GS. Indeed, their immunohistochemistry-staining pattern is broadly similar to that of our positive controls: the Moorfields/Institute of Ophthalmology cell line, which has been studied extensively.[10,11] We stress that this report serves as an initial description, and we propose that the cells migrating from surgical retinal explants that we have identified merit further characterization.

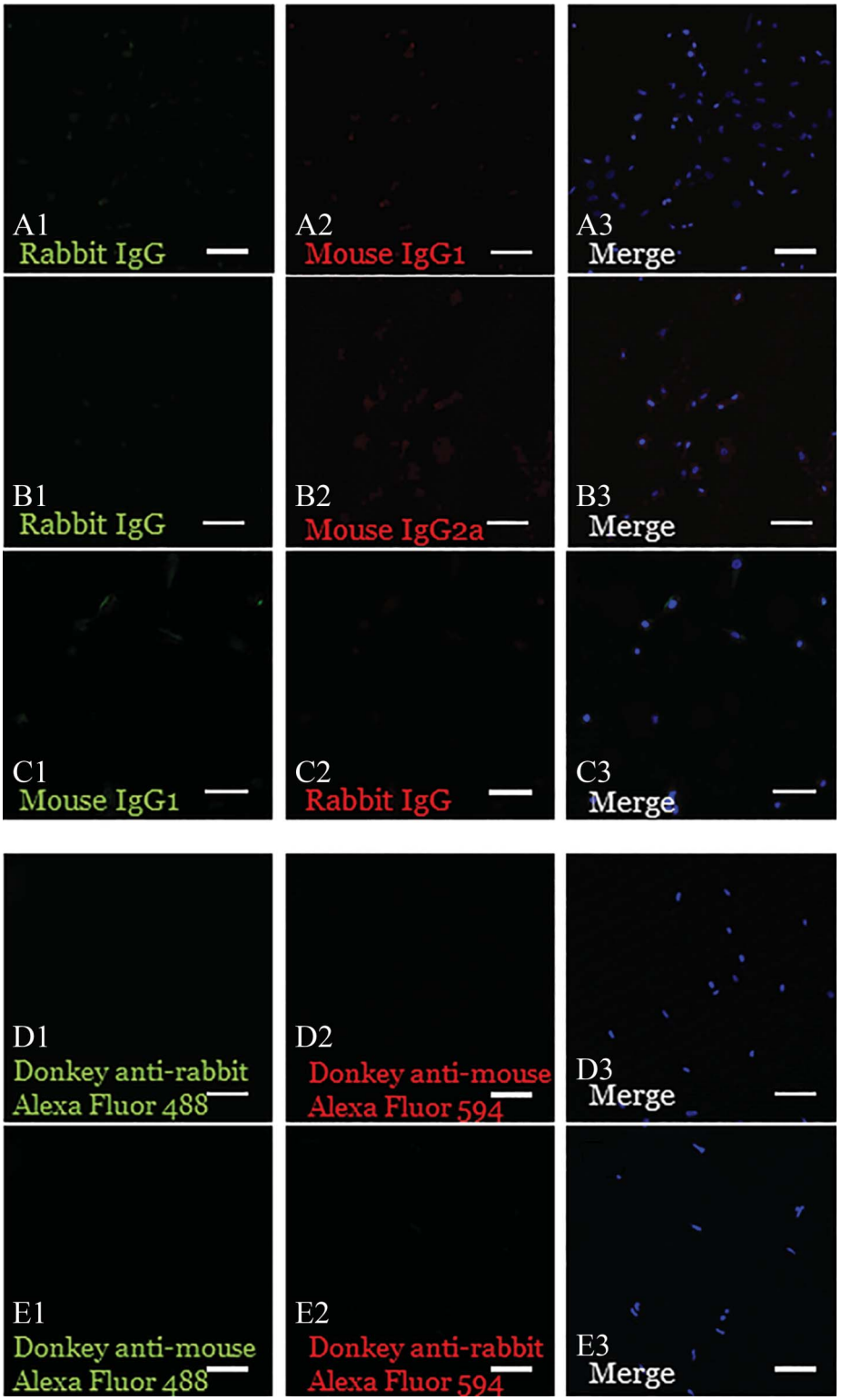

Fig. 4. Isotype and no primary antibody controls for immunofluorescent staining. **A1–A3.** Rabbit polyclonal IgG (dilution 1: 2,750) and mouse monoclonal IgG1 (dilution 1: 25) isotype controls. **B1–B3.** Rabbit polyclonal IgG (dilution 1: 550) and mouse monoclonal IgG2a (dilution 1: 125) isotype controls. **C1–C3.** mouse monoclonal IgG1 (dilution 1: 50) and rabbit polyclonal IgG (dilution 1: 1,375) isotype controls. Immunofluorescent staining in the absence of primary antibodies with (**D1–D3**) donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 594 secondary antibodies; (**E1–E3**) donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 594 secondary antibody. IgG, immunoglobulin G.

How can we account for the discrepancy between our findings (i.e., spontaneous production of migrating cells expressing progenitor markers) and those of human cadaveric studies (where growth factors seem to be required)? We hypothesize that our observations represent a tissue response to injury in the living human retina. For example, it is suggested that Müller cell proliferation is a stereotypical response to multiple

models of retinal injury.[3,7,8] The initiating event of rhegmatogenous retinal detachment in our patients seems to have been the traumatic event required to initiate this cellular response; alternatively—although perhaps less likely—sectioning of the retina during trimming of the flap tear may have been an inciting or exacerbating event. The migrating cells that we describe are presumed to represent in vitro some of the pathological mechanisms underlying the early tissue response to trauma/rhegmatogenous detachment. Although such mechanisms can lead to regeneration in certain animals, such as zebrafish,[3,8] in man they lead to repair processes such as proliferative vitreoretinopathy. Moreover, autologous transplantation of retinal explants collected from peripheral retina has recently been shown to be effective in treating recalcitrant macular hole.[21–23] One possible mechanism of closure after such transplantation is hypothesized to be through the production and integration of progenitor cells from explants.[21]

In conclusion, living donor human surgical retinal explants in tissue culture seem as a source of spontaneously migrating cells that express progenitor cell markers, including Pax6, Sox-2, CRX, nestin, and $p27^{kip1}$. In addition, these cells express proteins consistent with Müller cell lineage. Considering the plentiful potential supply of retinal explants in busy surgical units, these cells may provide an attractive alternative to cadaveric retinal cell culture techniques[11] (in much the same way as surgical explants provide an alternative to cadaveric explants for gene transduction studies).[15] Given that the Müller cells of multiple species have been reported to harbor stem cell–like properties, combined with previous descriptions from cadaveric studies of explanted retina from a similar retinal location,[10,11] and taken together with the findings of our immunohistological staining, we believe that it is most likely that the migrating cells we describe are derived from Müller cells. Furthermore, our observations support the assertion that the human retina possesses an innate potential for reentering the cell cycle to express progenitor cell markers. Although the regenerative potential of these cells remains to be explored, previous studies have identified potential pathways responsible for cell reprogramming and through which reprogrammed mammalian Müller cells may regenerate functional retinal neurons in vivo.[24–27] The natural history of retinal detachment suggests that the ultimate pathway of the process leading to the production of migrating progenitor cells in man—if unchecked—is gliosis and proliferative vitreoretinopathy.[28] However, we demonstrate that progenitor cells are produced from retinal explants in the absence of proliferative vitreoretinopathy, suggesting that the production of such cells may also be a feature of "normal" retinal repair. Furthermore, we hypothesize that it may ultimately be possible to redirect this process toward the pathway of regeneration seen in other species.[3,7,8]

Key words: retina, vitrectomy, retinal explant, stem cells, progenitor cells.

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