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Original Article

Biodegradable Polymer Composite Grafts Promote the Survival and Differentiation of Retinal Progenitor Cells

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ABSTRACT

Retinal progenitor cells (RPCs) are multipotent central nervous system precursors that give rise to all of the cell types of the retina during development. Several groups have reported that mammalian RPCs can be isolated and expanded in culture and can differentiate into retinal neurons upon grafting to the mature, diseased eye. However, cell delivery and survival remain formidable obstacles to application of RPCs in a clinical setting. Because biodegradable polymer/progenitor constructs have been shown to be capable of tissue generation in other compartments, we evaluated the survival, migration, and differentiation of RPCs delivered on PLLA/PLGA polymer substrates to the mouse subretinal space and compared these results to conventional injections of RPCs. Polymer composite grafts resulted in a near 10-fold increase in the number of surviving cells after 4 weeks, with a 16-fold increase in cell delivery. Grafted RPCs migrated into the host retina and expressed the mature markers neurofilament-200, glial fibrillary acidic protein, protein kinase C-α, recoverin, and rhodopsin. We conclude that biodegradable polymer/progenitor cell composite grafts provide an effective means of increasing progenitor cell survival and overall yield when transplanting to sites within the central nervous system such as the retina.
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INTRODUCTION

The two major clinical subtypes of retinal degeneration (RD) are retinitis pigmentosa and age-related macular degeneration (ARMD). A hallmark of these diseases is photoreceptor cell degeneration resulting in visual loss. No effective restorative treatment exists for either subtype. Recently, the transplantation of stem and progenitor cells has shown promise as a strategy for photoreceptor replacement [1–4]. Many mammalian tissues, including the retina, contain stem or progenitor cells that can be isolated, propagated, and grafted to animal models of retinal degeneration [3, 4]. The goal of these studies is to either replace or preserve the function of photoreceptors in the affected eye.

Previously, it has been reported that brain-derived progenitor cells can migrate and differentiate into cells expressing markers of mature neurons and glia when grafted to the retina of mice and rats with RD [3–9]. Despite incorporation into the host retina and morphological similarities to various retinal cell types, in each of these studies the transplanted cells failed to express retina-specific markers. In an attempt to overcome this hurdle, retinal progenitor cells (RPCs) have been isolated from two different derivatives of the embryonic eye cup: the ciliary epithelium and the neuroretina. These RPCs have the capacity to differentiate into photoreceptors and other cells of retinal lineage. However, poor survival of grafted cells remains a significant barrier to functional cell replacement.

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Similar problems have been seen in other cell-based therapies, such as intracerebral grafts in animal models [10] and patients with Parkinson's disease [11]. Moreover, bolus injection of cells into the subretinal space does not result in a well-organized photoreceptor layer of the type needed for high visual acuity.

We have applied tissue engineering techniques to this problem by using biodegradable polymers as a substrate for RPC grafts. Tissue engineering has arisen, in part, to address the shortage of tissues and organs available for transplantation [12]. It has shown promise in the repair of bladder [13], cartilage [14], and skin [15] defects and is being evaluated for a range of additional clinical applications. Moreover, poly (lactic-*co*-glycolic acid) (PLGA)/poly (L-lactic acid) (PLLA) polymers exhibit a high degree of biocompatibility in the brain [16] and spinal cord [17]. Tissue engineering does not yet allow the construction of a functional retina de novo, but polymers have the potential to address several critical, unsolved problems in retinal transplantation. We hypothesize that an organized graft, containing aligned and polarized cells, is more readily achieved by seeding the cells onto a PLLA/PLGA polymer scaffold before transplantation. The polymer construct could also increase control over graft size and placement. Importantly, donor cell survival may be improved because cell death, leakage, and migration from the injection site occur when RPCs are delivered as a single-cell suspension [4].

Biodegradable polymers are attractive in tissue engineering applications for several reasons, including availability and ease of manufacture. The polymers can be easily processed into a variety of structures, and degradation can be readily controlled. These constructs have FDA approval for use in several applications [18]. Here we develop a biodegradable polymer/retinal progenitor composite graft that provides an effective method for transplantation of progenitor cells to the subretinal space of RD mice by significantly improving survival, control of delivery, and cellular differentiation compared with injection of dissociated cells.

Materials and Methods

Scaffold Fabrication

Polymers were constructed using a 50/50 blend of PLGA lactic acid to glycolic acid ratio of 75:25 (mean, ~45,000 g/mol; Boehringer Ingelheim GmbH, Ingelheim, Germany, http://www.boehringer-ingelheim.com) and PLLA (mean, ~100,000 g/mol; Polysciences, Warrington, PA, http://www.polysciences.com) dissolved in 5% wt/vol dioxane solution. We used a modification of the solid-liquid-phase separation technique described by Schugens et al. [19]. The solution (0.3–0.5 ml) was added to a glass slide and allowed to spread uniformly across the slide. The slide was then placed on ice. After 1 minute, a 20-gauge piece of copper wire, which had been sitting in dry ice, was touched to the surface of the slide to initiate nucleation of dioxane crystals. The growth front spread along the surface of the slide and then moved normal to the slide, growing toward the solution/air interface. Once the dioxane had completely solidified, the slide was transferred to a freezer at –20°C for at least 1 hour. Slides were then placed on a lyophilizer to sublimate the dioxane, leaving behind the polymer scaffolds whose pore structure was a direct artifact of the freezing process.

The porosity was estimated for the samples by a simple calculation of the ratio of density of a series of six samples via their weight in air divided by their volume to the density of the polymer as reported by the manufacturer. The pore size data were determined by measuring 40 to 50 pores at the surface over a series of six scaffolds. Cross sections of the scaffolds in scanning electron microscopy (SEM) were used to confirm that the pores were generally uniform in diameter from the surface through the thickness of the samples.

Donor Cell Line

RPCs harvested from the retina of postnatal day 1 enhanced green fluorescence protein mice (C57BL/6 background; kind gift of Dr. Okabe, University of Osaka, Osaka, Japan) were isolated and maintained in culture as previously described [4]. Briefly, whole-retina homogenates were incubated in 0.1% collagenase, and single-cell suspensions were obtained. Dissociated cells were then cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with N-2 (GIBCO, Grand Island, NY, http://www.invitrogen.com) and 20 ng/ml of epidermal growth factor (EGF). The neurospheres that were generated could in turn be dissociated and subcultured to regenerate new spheres.

Differentiation and Characterization of Donor Cell Line

To examine the differentiation of green fluorescent protein (GFP)–expressing RPCs in vitro, RPC spheres were incubated with trypsin for 1 minute to generate a single-cell suspension. Cells (1.0×10^3) were plated on eight-well laminin-coated chamber slides (BD Biosciences, San Diego, http://www.bdbiosciences.com) in DMEM/F12 media supplemented with 10% fetal bovine serum and were fixed with 4% paraformaldehyde at 1 day and 2 weeks after plating. The cells were blocked in 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) plus 0.2% Triton-100 (Sigma-Aldrich) and then incubated for 2 hours with primary antibody for Ki67 (1:100, cell proliferation marker; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com), nestin (1:1, immature neuronal marker; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, http://www.uiowa.edu/~dshbwww), glial fibrillary acidic protein (GFAP) (1:50, astrocytic marker, DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com), MAP-2, or neurofilament-200 (NF-200) (1:500 and 1: 1000, respectively, neuronal markers; Sigma-Aldrich), protein kinase C (PKC)-α (1:200, bipolar cell marker; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), 2D4 (1:500, rhodopsin marker; kind gift of Dr. R. Molday, University of British Columbia, Vancouver,

British Columbia, Canada), and recoverin (1:1000, photoreceptor marker; Chemicon, Temecula, CA, http://www.chemicon.com). After rinsing in phosphate-buffered saline (PBS), samples were incubated in Cy3-conjugated species-specific immunoglobulin G (IgG) (1:800) for 1 hour. Samples were rinsed again and then coverslipped in poly vinyl alcohol (PVA)-Dabco with DAPI and viewed under fluorescent illumination.

EGF Antibody Staining of Polymers

To evaluate whether growth or survival factors could be sequestered in the polymer substrate, we stained the polymer for antibodies against EGF. We evaluated grafts containing cells at 1, 2, and 4 weeks after transplantation and acellular polymers in vitro after exposure to EGF-containing media. Polymers were sterilized overnight in 70% ethanol. After rinsing three times with Hanks' balanced salt solution, polymers were incubated with DMEM media with EGF (10 μg/ml), fixed in 4% paraformaldehyde (PFA), and sectioned at day 7 ($n = 6$). As a control, the polymer treated with 5% PVA (Sigma-Aldrich) for 5 hours was also cultured with EGF. Sections were blocked in 1% BSA (Sigma-Aldrich) plus 0.2% Triton-100 (Sigma-Aldrich) and then incubated for 2 hours with primary antibody of EGF (1:20; R&D Systems Inc., Minneapolis, http://www.rndsystems.com) for 2 hours, followed by reaction with species-specific IgG conjugated to Cy3 (1:800) for 1 hour. Samples were rinsed again and then coverslipped in PVA-Dabco viewed under fluorescent illumination.

Graft Preparation

RPCs were seeded onto polymer scaffolds that had been cut into 10 × 10-mm squares and sterilized overnight in 70% (vol/vol) ethanol. The polymers were incubated with 5 ml laminin solution (0.005 mg/ml) for 60 minutes at 37°C and then washed three times in PBS in a six-well plate. After laminin treatment, 4 ml of culture media was added to the six-well plate. After 60 minutes in a 37°C incubator, most of the culture media was removed from the six-well plate, and RPCs (1.0×10^6 cells) were dispersed on the polymer coated with the laminin and incubated for an additional 60 minutes. After incubation, 4 ml of culture media was added to the six-well plates. Three days after initial seeding, most of the culture media was removed, and polymers were turned over to seed RPCs onto the obverse side, as described above. Seven days after initial seeding, the polymer/RPC composites were cut into rectangular pieces of 0.5 × 0.3 mm using a Mcllwain tissue chopper (Mickle Laboratory Engineering, Gomshell, U.K.).

Differentiation and Characterization of Donor Cell Line on Polymer Composite Graft

To examine the differentiation of donor cells before transplantation, some composites were placed into a 24-well culture dish and then fixed with 4% paraformaldehyde for 1 hour. The composites were blocked in 1% BSA (Sigma-Aldrich) plus 0.2% Triton-100 (Sigma-Aldrich) and then incubated for 2 hours with primary antibodies against Ki67, nestin, Map2, GFAP, PKC-α, recoverin, and rhodopsin (see above) for 2 hours, followed by reaction with species-specific IgG conjugated to Cy3 (1:800) for 1 hour. Samples were rinsed again and then coverslipped in PVA-Dabco with DAPI and viewed under fluorescent illumination.

Recipient Animals

Recipient mice were C57/BL/6 (survival times: 1 week, $n = 20$; 2 weeks, $n = 15$; 4 weeks, $n = 15$) and rhodopsin knockout mice ($rho^{-/-}$) (survival times: 1 week, $n = 6$; 2 weeks, $n = 10$; 4 weeks, $n = 10$), grafted at 4 to 8 weeks of age (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org). All animals were treated in accordance with guidelines defined by the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Transplantation

Recipient mice were treated with polymer/RPC composite grafts or single-cell injections, both placed into the subretinal space under general (ketamine/xylazine) and topical (proparacaine) anesthesia as previously described [4]. Transplantation was performed under direct observation using a binocular surgical microscope and viewed through a dilated pupil (topical tropicamide 1%). At the conclusion of all surgeries, fundus examination was performed via surgical microscope to confirm successful graft placement.

Transplantation of RPC/Polymer Composite Graft

A conjunctival incision and small sclerotomy were made using an extrafine disposable scalpel. The polymer composite pieces (each containing ~16,000 cells) were inserted through the sclerotomy into the subretinal space using fine #5 Dumont forceps (Fine Science Tools, North Vancouver, British Columbia, Canada, http://www.finescience.com). The polymer could be easily imaged through the pupil using either fluorescent or standard illumination.

Single-Cell Transplantation

A small sclerotomy was made and cells were injected into the subretinal space using a glass pipette (internal diameter, 150 μm) attached to a 50-μl Hamilton syringe via polyethylene tubing. Two microliters of GFP donor cell suspension, containing 16,000 single cells, were injected into the subretinal space. During transplantation, the intraocular pressure was reduced by making a small puncture through the cornea.

Tissue Preparation

At 1, 2, and 4 weeks after transplantation, the grafted eyes were enucleated and immersion fixed with 4% paraformaldehyde, followed by cryoprotection with 20% sucrose. Eyes were sectioned at 12 μm on a cryostat. Sections were stained with antibodies as described above. Some sections were also stained by hematoxylin and eosin.

Cell Counting Methods

One week after culture, composites were cut into 0.3 × 0.5-mm pieces with a tissue chopper. Every other piece was fixed in 4% PFA and sectioned to assess the total cell number before transplantation. Alternate pieces were transplanted into the subretinal space. A single-cell suspension of RPCs was also transplanted into the subretinal space as a control. Eyes were enucleated, fixed in 4% PFA, and sectioned at days 0, 1, and 2 and 4 weeks. Samples were rinsed again and then coverslipped in PVA-Dabco with DAPI and viewed under fluorescent illumination. Both GFP and DAPI$^+$ cells were counted in one out of every four sections. Experimental and control cell numbers were then compared, and statistical analysis was performed using a Student's *t*-test.

Evaluation of TUNEL$^+$ Cells with Different Procedures

To examine the effect of traumatic cell death in the different transplantation methods, we counted the number of TUNEL$^+$ cells under single-cell injection and polymer construct conditions. RPC spheres were incubated with trypsin for 1 minute to generate a single-cell suspension. Cells (1.0×10^3) were plated on eight-well laminin-coated chamber slides (BD Biosciences) in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS) using one of two methods: either a pulled-glass needle as used in the conventional method of subretinal transplantation ($n = 6$) or a 200-μl pipette tip (Falcon, BD Biosciences) as used for seeding polymers ($n = 6$). We also used the same method to seed polymers with RPCs ($n = 6$). Cells were cultured under differentiation conditions with 10% FBS and were fixed with 4% paraformaldehyde at 3 days, with the polymers sectioned as previously described. The cells were blocked in 1% BSA (Sigma-Aldrich) plus 0.2% Triton-100 (Sigma-Aldrich), and TUNEL staining was then performed using a TUNEL staining kit (Roche Diagnostics, Manheim, Germany, http://www.roche-applied-science.com). Every sample was rinsed again and then coverslipped in PVA-Dabco with DAPI and viewed under fluorescent illumination. DAPI$^+$ cells and TUNEL$^+$ cells were counted under each condition, and statistical analysis was performed using a Student's *t*-test.

Results

Formulation of Polymer Scaffolds

Scaffolds were fabricated using the solid-liquid-phase separation technique leading to pores oriented normal to the plane of the scaffold as desired for mimicking the polarized cytoarchitecture of the retina. The pore structure was largely uniform, with diameters of approximately 35–50 μm. The polymer solution (PLGA in dioxane) was cast onto slides and frozen on ice at 0°C, leading to the precipitation of the polymer solute from the solid dioxane phase. The dioxane was then sublimated, leaving the PLGA with a unique pore architecture as a direct artifact of the dioxane crystallization. A wide range of pore architectures can be obtained by controlling the concentration of dioxane, the amount of undercooling, and the thermal gradient. Our goal was to seed the scaffolds with RPCs; thus, relatively large, oriented pores were desired. We used very little undercooling (11°C), coupled with the promotion of nucleation at the glass surface via contact with a copper coil at –80°C. The solid phase was observed to grow along the glass slide and then normal to the glass slide. After sublimation of the dioxane, we produced polymers with a large, oriented, and reproducible pore structure (Figs. 1A–1C).

Characterization of Donor Cells In Vitro

When grown on conventional substrates in media supplemented with EGF, GFP-transgenic RPCs exhibited high levels of endogenous green fluorescence (Fig. 2A) and maintained an undifferentiated state characterized by ubiquitous Ki67 and nestin immunoreactivity (Figs. 2B, 2C). Cells could be maintained in this state for up to 1 year. To examine differentiation in vitro, media without EGF was supplemented with 10% FBS. After 2 weeks of culture under differentiation conditions, the cells were analyzed immunocytochemically. The number of Ki67$^+$ cells markedly decreased (data not shown), and subpopulations expressed GFAP, Map2, PKC-α, recoverin, or rhodopsin. These markers are consistent with differentiation into rod photoreceptors, bipolar cells, and Muller glia, all of which are known to be born late in retinogenesis. Interestingly, these immunopositive cells also showed morphological evidence of differentiation into rod photoreceptor and bipolar cell types (Fig. 2). No immunocytochemical or morphological evidence of early-born cell types (e.g., cones or retinal ganglion cells) was observed. These data indicate that RPCs derived from neonatal mice have the intrinsic potential to differentiate into late-born retinal cell types.

Attachment and Incorporation of RPCs onto the Polymer Substrate

The solid-liquid-phase separation technique using dioxane was able to generate polymers with the appropriate pore structure for the seeding of RPCs. The pore architecture can be seen in SEM images (Figs. 1A–1C) before the addition of RPCs. The scaffolds are approximately 95% porous, and the pore size is estimated to be 35–50 μm in diameter. The parameters of the seeding procedure described in this work were developed to ensure the maximum seeding density without induction of cell death. We used a two-phase procedure in which one side of the polymer was seeded first and the other 3 days later, followed by an additional 4 days in culture. We determined that this method leads to a composite graft in which RPCs were fully incorporated yet not overcrowded such that significant cell death could occur. The seeded polymers are shown in SEM images (Figs. 1D, 1E) and under GFP illumination (Figs. 1F, 1G).

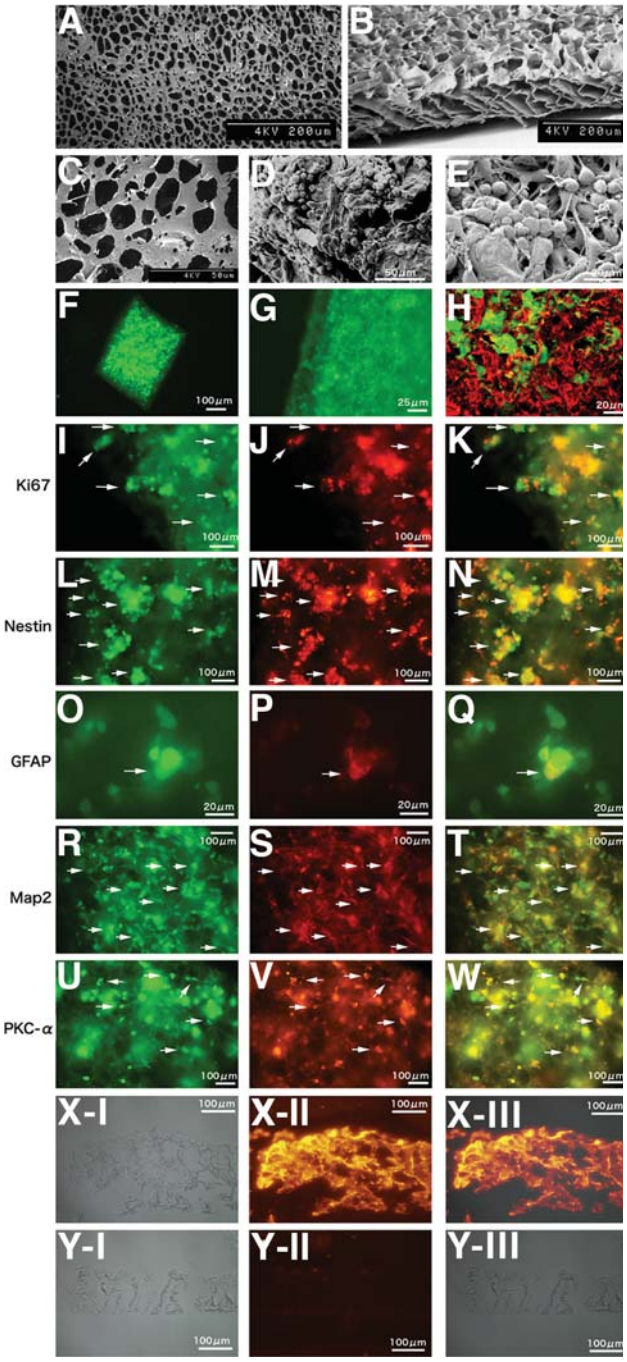

Figure 1. Polymer structure, differentiation, and characterization of donor cell line on polymer composite graft. Scanning electron microscope of the polymer substrate **(A, B, C)** before and **(D, E)** after seeding with RPCs. The polymer/RPC composite grafts were cut into rectangular pieces of 0.5 × 0.3 mm and viewed under fluorescein isothiocyanate illumination under **(F)** low and **(G)** high magnification. The polymer composite grafts were cut into smaller pieces for transplantation, and some of these pieces were then analyzed immunocytochemically using epifluorecent microscopy. Constitutive GFP expression **(I, L, O, R, U)**, antibody/Cy3 immunoreactivity, Ki67 **(J)**, nestin **(M)**, GFAP **(P)**, Map2 **(S)**, and PKC-α **(V)**, and merged images **(K, N, Q, T, W)**. Arrows indicate cells coexpressing these labels. **(I–K):** GFP$^+$ RPCs coexpressed Ki67 on the composite graft. **(L–N):** GFP$^+$ RPCs coexpressed nestin on the polymer composite graft. Some cells coexpressed **(O–Q)** GFAP, **(R–T)** Map2, or **(U–W)** PKC-α on the composite graft. Most of the RPCs on the composite grafts continued to express Ki67 and nestin, although some cells now expressed neuronal and astrocytic markers before transplantation. **(H):** Polymer composite grafts were EGF$^+$ at 1 week after transplantation to subretinal space in vivo. Polymer incubated with media containing EGF were EGF$^+$ **(X-I-X-III)**, whereas polymers pretreated with 5% poly vinyl alcohol before EGF incubation were EGF$^-$ **(Y-I-Y-III)** after 1 week in vitro. Abbreviations: EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; PKC, protein kinase C; RPC, retinal progenitor cell.

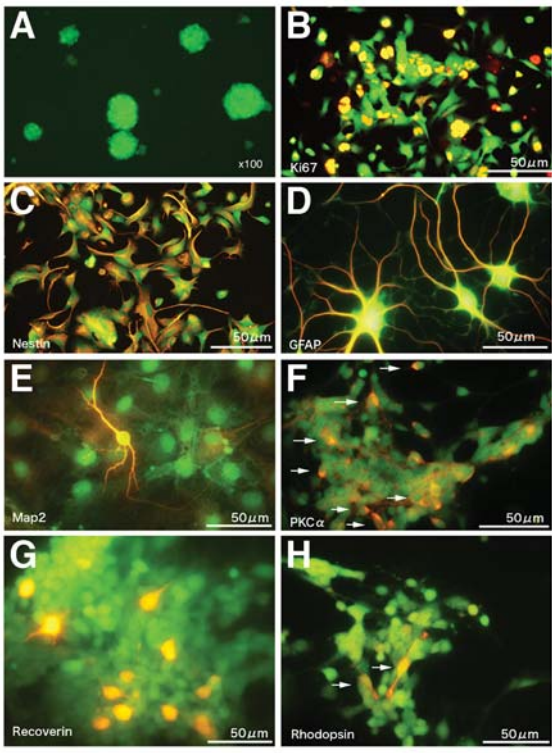

Figure 2. Differentiation and characterization of donor cells in vitro. RPCs formed GFP$^+$ neurospheres when grown in serum-free media supplemented with EGF. **(A):** Viewed under fluorescein isothiocyanate illumination. RPCs were plated on eight-well slides coated with laminin and were cultured in the absence of EGF and the presence of 10% fetal bovine serum for **(B, C)** 1 day or **(D–H)** 14 days. The cells were stained for **(B)** Ki67, **(C)** nestin, **(D)** GFAP, **(E)** Map2, **(F)** PKC-α, **(G)** recoverin, and **(H)** rhodopsin. At day 1, cells expressed **(B)** Ki67 and **(C)** nestin. At day 14, some cells differentiated morphologically into specific cell types expressing **(D)** GFAP, **(E)** Map2, **(F)** PKC-α, **(G)** recoverin, and **(H)** rhodopsin. Abbreviations: EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; KC, protein kinase C; RPC, retinal progenitor cell.

Characterization of Donor Cells on Polymer Scaffolds Before Grafting

Polymer/RPC composites were analyzed immunocytochemically to determine whether culturing RPCs on a PLGA substrate induces changes in gene expression indicative of differentiation. Polymer/RPC composites were cultured for 7 days and then cut into 0.5 × 0.3-mm fragments, as used for transplantation, and examined for the cellular markers listed above. Most RPCs expressed Ki67 and nestin (Figs. 1I–1N), although at a lower level than that seen when the same cells were grown under identical conditions but without the PLGA substrate. A subpopulation of cells expressed the more mature markers GFAP, Map2, and PKC-α (Figs. 1O–1W); however, there was no evidence for expression of the retina-specific markers recoverin or rhodopsin. These data indicate that most of the RPCs cultured on a PLGA scaffold remain relatively undifferentiated before grafting, whereas a subset expresses early markers of more mature neurons or glia.

Biodegradable Polymers Stained for EGF In Vivo and In Vitro

Sections were stained with EGF antibody at 1, 2, and 4 weeks after transplantation to subretinal space of B6 mice. We found EGF^+ staining throughout the polymer at all time points (Fig. 1H). We also found EGF^+ staining of the polymer after 1 week in culture with EGF (Figs. 1X-I-X-III). No EGF^+ staining was detected when the polymer was treated with 5% PVA (Figs. 1Y-I-Y-III). These results suggest that the biodegradable polymer used here can adsorb the key cell survival factor EGF in both an in vitro model and in an in vivo transplantation model. Furthermore, this EGF adsorption is dependent on the hydrophobic properties of the polymer, as treatment with PVA eliminates this effect. The presence of the EGF, and perhaps other factors, on the biodegradable polymer may in part underlie the increased cell survival seen in these experiments.

The Fate of RPCs Transplanted as a Polymer Composite Graft to the Subretinal Space

Survival and integration (defined as morphological incorporation) of grafted RPCs was evaluated by fundus examination as well as immunohistochemical analysis of tissue sections (Figs. 3A-3H). Intraocular GFP^+ profiles were identified at 1, 2, and 4 weeks after transplantation using in vivo fluorescence microscopy (Figs. 3E–3H). Survival and integration of grafted RPCs in the retina of nonimmunosuppressed host animals were confirmed by histological analysis. In syngeneic C57/Bl6 mice and $rho^{-/-}$ mice, surviving RPCs were found in all recipient eyes at all three points (total, 76/76; 100%). GFP-expressing RPCs frequently migrated into the host retina, where they showed signs of morphological integration. At 1, 2, and 4 weeks after transplantation, integrated RPCs were found in 83%, 100%, and 90% of adult $rho^{-/-}$ mice and 85%, 87%, 87% of adult C57/Bl6 mice, respectively (Table 1).

Polymer Degradation

The breakdown of RPC-seeded PLGA scaffolds was examined in cryosections and compared at 1, 2, and 4 weeks after transplantation to the subretinal space. Gradual breakdown of the polymer component of the composite grafts was seen over the course of this period (Figs. 3J–3L). Pores progressively increased in size and merged, such that at 4 weeks, very little of the polymer scaffold remained. Although the average overall thickness did not change substantially, degradation was observed as an increase in the pore size, such that large channels were observed in the polymers at 2 and 4 weeks after implantation (Figs. 3K, 3L).

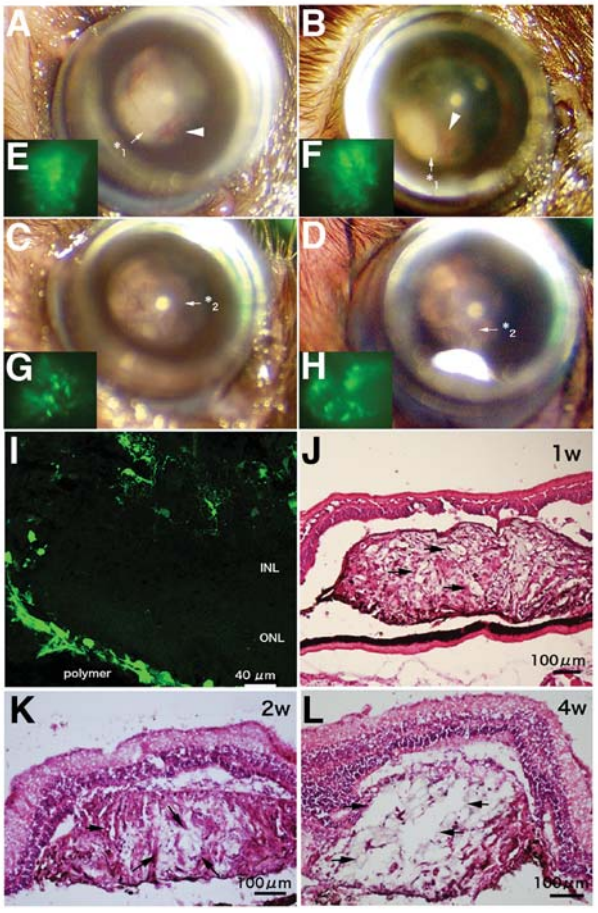

Figure 3. Fundus examination after composite graft transplantation. Fundus pictures of the same C57/Bl6 mouse at **(A)** 2 weeks and **(B)** 4 weeks. Fundus pictures of the same $rho^{-/-}$ mouse at **(C)** 2 weeks and **(D)** 4 weeks. The arrowheads show the optic nerve head, and arrows show the same retinal vessel. **(E–H):** In each eye, surviving RPCs were confirmed to be GFP^+ by fundus examination via fluorescent microscopy. **(I):** A large number of RPCs migrated into the host retina. Hematoxylin and eosin staining after operation at **(J)** 1, **(K)** 2, and **(L)** 4 weeks. **(J–L):** These polymers in the subretinal space were gradually degraded at 1, 2, and 4 weeks, with pores increasing in size over this period (arrows). Abbreviation: RPC, retinal progenitor cell.

Table 1. Survival and integration of grafted retinal progenitor cells in the eye of nonimmunosuppressed host animals at 1, 2, and 4 weeks after transplantation

	Recipient age	No.	1 wk	2 wks	4 wks
C57/BL6	6–8 wks	50	$n = 20$	$n = 15$	$n = 15$
		Survival/integration	$n = 20/17$	$n = 15/13$	$n = 15/13$
$Rho^{-/-}$ mice	4–6 wks	26	$n = 6$	$n = 10$	$n = 10$
		Survival/integration	$n = 6/5$	$n = 10/10$	$n = 10/9$

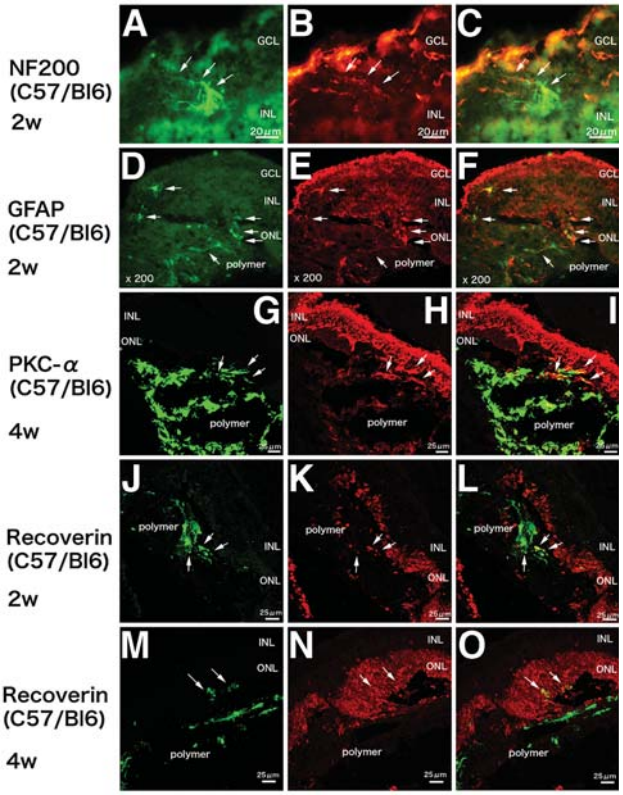

Figure 4. Migration and differentiation of RPCs from polymer composite grafts into C57/Bl6 mice retina. Epifluorescent **(A–F)** and confocal **(G–O)** images of the expression of neural and photoreceptor markers by RPCs after polymer composite grafting to the eye of normal adult C57/Bl6 mice, seen at 2 and 4 weeks after grafting; **(A, D, G, J, M)** constitutive GFP expression, **(B)** antibody/Cy3 immunoreactivity for NF200, **(E)** GFAP, **(H)** PKC-α, **(K, N)** recoverin, and **(C, F, I, L, O)** merged images. **(A–C):** NF-200–coexpressing RPCs extended neural fibers. **(D–F):** GFAP-coexpressing RPCs migrated from the composite graft into the host retina, and some RPCs in the polymer also expressed GFAP. **(G–I):** PKC-α–coexpressing RPCs were found in the host inner nuclear layer. Recoverin-coexpressing RPCs were found in the **(J–L)** composite graft and the **(M–O)** host retina of C57/Bl6 mice at 2 and 4 weeks after transplantation. Abbreviations: GFAP, glial fibrillary acidic protein; PKC, protein kinase C; RPC, retinal progenitor cell.

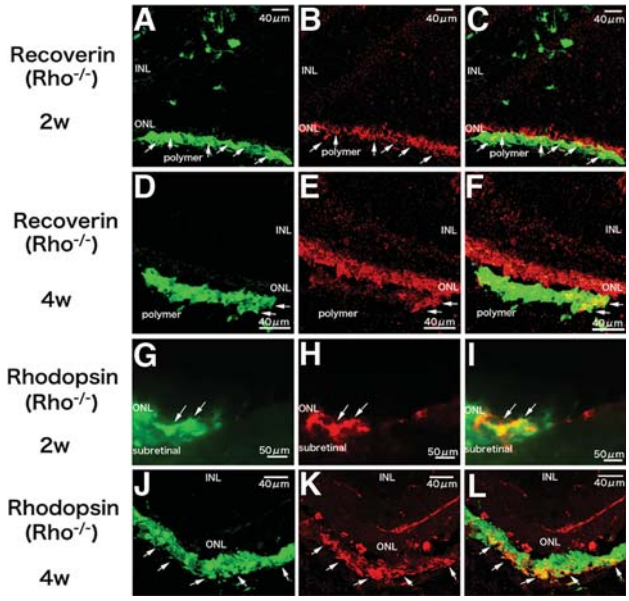

Figure 5. Migration and differentiation of RPCs from polymer composite grafts into the $rho^{-/-}$ retina. Confocal **(A–F, J–L)** and epifluorescent **(G–I)** images of the expression of neural and photoreceptor markers by RPCs after polymer composite grafting to the eye of adult $rho^{-/-}$ mice at 2 and 4 weeks after grafting. Constitutive GFP expression **(A, D, G, J)**, antibody/Cy3 immunoreactivity for recoverin **(B, E)**, rhodopsin **(H, K)**, and merged images **(C, F, I, L)**. Recoverin-coexpressing RPCs were found in the retina of $rho^{-/-}$ mice at **(A–C)** 2 weeks and **(D–F)** 4 weeks after transplantation. Rhodopsin-coexpressing RPCs were found in $rho^{-/-}$ mice at **(G–I)** 2 weeks and **(J–L)** 4 weeks. Abbreviation: RPC, retinal progenitor cell.

Migration and Differentiation of Cells Delivered as Polymer/RPC Composite Grafts

At 1, 2, and 4 weeks after transplantation, RPCs migrated into retinal laminae adjacent to the graft and showed morphological evidence of neuronal differentiation (Figs. 3I, 4). GFP^+ donor cells coexpressed several markers indicative of phenotypic maturation, including NF200, GFAP, PKC-α, recoverin, and rhodopsin (Figs. 4, 5). Expression of these markers increased after transplantation, with rhodopsin and recoverin becoming more intense and widespread by the 4-week time point. Interestingly, rhodopsin was expressed by RPCs grafted to $rho^{-/-}$ recipients, a finding not seen in previous work using single-cell suspension grafts in this model [4].

Cell Survival in Polymer/RPC Composite Grafts Versus Single-Cell Suspensions

RPCs were counted before grafting and at 1, 2, and 4 weeks after transplantation to the subretinal space as a composite graft or single-cell suspension. Before transplantation, each polymer graft contained approximately 6,000 cells (mean ± SD, 15,800 ± 672; $n = 7$), and this same number of cells was used for the single-cell suspension grafts. Because a significant drawback of single-cell suspensions is reflux of cells from the injection site, we also counted the number of cells present immediately after

injection. An average of 9,232 cells (57.7% of those grafted) were still present in recipients analyzed at day 0 ($n = 6$), indicating that 43.3% of the grafted cells were either lost due to reflux or did not survive the transplantation procedure. We then assessed the number of surviving cells in the polymer composite grafts at 1, 2, and 4 weeks after transplantation and found 91% ($n = 6$), 96% ($n = 6$), and 78% ($n = 6$) survival at these time points, respectively (Fig. 6A). Results for single-cell suspension grafts, calculated relative to the day-0 result (9,232), showed survival of 12.7% ($n = 6$), 9.9% ($n = 6$), and 8.1% ($n = 6$) at 1, 2, and 4 weeks (Fig. 6A). These data show that the percent survival of RPCs delivered as a polymer composite graft is approximately 10-fold higher than cell-suspension grafts after 4 weeks ($p < .01$). Moreover, if one includes in the calculation the number of cells that die or leak out during the grafting procedure, a 16-fold improvement in cell delivery to the subretinal space is obtained using a polymer composite graft.

TUNEL$^+$ Cells Are Greatly Reduced with Polymeric Delivery Methods

We assessed the number of TUNEL$^+$ cells in cell culture preparations that were analogous to the transplantation methods used in this study. The preparation that used a conventional glass needle to seed (rather than inject) RPCs resulted in an extremely high rate of cell death (57.8% TUNEL$^+$) at 3 days after seeding onto culture slides (Fig. 6B). In contrast, when we used the pipette method of seeding onto either culture slides, or polymer substrates, the rate of TUNEL$^+$ staining was decreased to 1.56% and 14.9%, respectively. These results indicate that traumatic cell death associated with cell injection is greatly reduced by using a large-bore pipette rather than a small-bore glass needle and that cell death is approximately 9.5% higher when seeding onto the biodegradable polymers compared with standard culture-treated slides.

Discussion

Efficiency of cell delivery, and the related problem of cell survival, have posed a serious impediment to effective transplantation within the central nervous system (CNS) since the earliest attempts at brain repair. This has been documented in studies of neural transplantation for Parkinson's disease, where populations of cells secreting dopamine or its precursors are grafted to the striatum as a means of ameliorating the behavioral deficits caused by the loss of dopaminergic neurons in the substantia nigra of the host [20, 21]. The work of Brundin et al. [22] has demonstrated that approximately 90% of grafted fetal mesencephalic neurons do not survive the transplantation procedure itself. This fact alone has made clinical application of this technology extremely difficult because up to four donor embryos are needed to provide a sufficient number of grafted cells for each hemisphere of the brain of the Parkinsonian patient. This issue has been less extensively studied in the setting of retinal transplantation, but it is clear from the literature that the loss of grafted cells, either by cell death or leakage, remains a significant challenge to widespread cell replacement in this CNS compartment as well. Reflux is a persistent aspect of the problem, particularly when delivering a bolus injection of cells to the subretinal space. The data we present here demonstrate that the use of a biodegradable polymer decreases the number of cells lost to reflux by almost 50%. With respect to leakage of cells from the subretinal space to the choroid or vitreous, this phenomenon is also problematic because of the potential for ectopic cells to initiate additional complications such as tractional retinal detachment, obstructed trabecular outflow, or intraocular inflam-

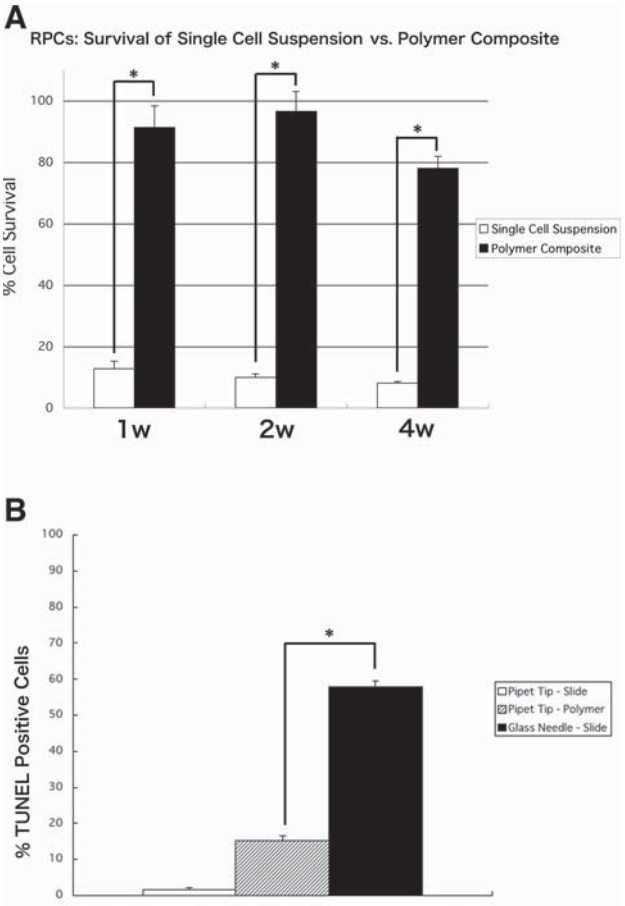

Figure 6. Integration and cell survival of RPCs. Comparison of polymer composite grafts and single-cell suspensions. **(A):** Cell numbers were compared with composites before transplantation and in eyes after composite transplantation. At 1, 2, and 4 weeks after transplantation, 91% ($n = 6$), 96% ($n = 6$), and 78% ($n = 6$) of the cells survived in the eye. To examine the number of cells transplanted, cell numbers at 1, 2, and 4 weeks were compared with cell numbers at day 0. At 1, 2, and 4 weeks after single-cell suspension transplantation, 12.7% ($n = 6$), 9.9% ($n = 6$), and 8.1% ($n = 6$) survived in the retina. These data show that polymer composite grafts can deliver 10-fold more cells compared with single-cell suspension transplantation. *$p <$ .01; Student's t-test. **(B):** The numbers of TUNEL$^+$ cells in the group injected using the conventional method with a glass needle (glass needle slide), with a plastic pipette tip (pipette tip slide), or using the pipette to seed the polymer composite graft (pipette tip polymer) were 57.8%, 1.56%, and 14.9% at 3 days. *$p < .01$, Student's t-test. Abbreviation: RPC, retinal progenitor cell.

mation, all of which would pose serious threats to vision. In terms of donor cell death, this process continues unabated during the first 4 weeks when using a single-cell suspension graft, whereas use of the polymer substrate decreases cell loss by more than ninefold. Overall, the use of polymer composite grafts results in an increase in cell delivery of nearly 16-fold over cell suspensions.

Recent transplantation studies have demonstrated that neural progenitor cells have the capacity to migrate into the immature, diseased, or injured retina and differentiate into cells that morphologically resemble retinal neurons. However, evidence to date indicates that brain-derived neural progenitor cells, as well as the less-mature embryonic stem cells, fail to differentiate into opsin$^+$ photoreceptors when grafted to the adult mammalian retina [3, 5–9, 23, 24]. In contrast, it has been demonstrated that neural progenitors derived from the retina (i.e., RPCs) can differentiate into cells of retinal lineage, including opsin$^+$ photoreceptors, both in vitro and in vivo. After transplantation of RPCs to a mouse model of retinal degeneration, recipients showed improved light sensitivity [4]. Nevertheless, several challenges to the effective application of this treatment strategy remain, including the control of cell delivery, survival of grafted RPCs, and efficiency of differentiation into photoreceptors. The present study demonstrates that each of these parameters can be significantly improved through the use of biodegradable substrates to deliver retinal progenitor cells to the retina.

Our experiments in vitro and in vivo shed light on the mechanisms underlying increased cell survival through polymeric delivery. Our in vitro modeling of the transplantation procedure demonstrates that significant cell death occurs when delivering progenitor cells through the small-bore glass needle typically used for subretinal transplantation. The use of a large-bore pipette tip (as used in the polymer seeding) greatly reduces this cell death.

The mechanism underlying the increased survival of grafted cells at extended time points is more difficult to determine. The evidence we present showing that the scaffold is capable of growth factor adsorption such as EGF is one possible explanation. As this binding takes place in both the in vitro model as well as the in vivo experimental paradigm under study here, the continued availability of the survival factor EGF (and likely other proteins) suggests a further role for the polymer in promoting the survival of grafted progenitor cells. Interestingly, the concentration of EGF seems to be at survival (<10 μg/ml) rather than mitogenic levels, as proliferation of the progenitor cells was not detected after transplantation (data not shown). These results point to the use of modified polymer substrates in the development of cell delivery systems.

The use of a polymer composite graft also seems to promote the differentiation of RPCs toward rod photoreceptors. Whereas we were previously unable to demonstrate the expression of rod photoreceptor markers after bolus injection of RPCs to the rho$^{-/-}$ subretinal space, in the present study, RPCs delivered to these same hosts as polymer composite grafts matured into cells expressing both recoverin and the rod-specific marker rhodopsin. This finding is consistent with our other work showing an increased tendency toward differentiation of RPCs cocultured on a PLGA/PLLA substrate [25].

It is widely appreciated that biodegradable polymers provide an excellent substrate for cell transplantation and tissue engineering [12, 26–30]. For instance, biodegradable polymers cultured with bone marrow–derived cells have been used to promote regeneration of the diseased skeletal system [29, 30]. In another report, biodegradable polymers were seeded with neural stem cells and the composite grafts transplanted to the injured spinal cord, resulting in regeneration of host axons with concomitant behavioral recovery [27]. To our knowledge, however, there has been no prior report comparing the survival of cells delivered as a single-cell suspension versus as a polymer composite graft.

In addition to the benefits enumerated above, the use of polymer composite grafts confers additional advantages in the setting of retinal repair. For instance, placement of a graft in the subretinal space requires the formation of a focal, transient detachment of the retina. The position of this detachment can shift during the time the retina is elevated, potentially resulting in suboptimal graft placement. Because of their physical properties, polymer composite grafts provide stable graft placement relative to single-cell suspensions, hence ensuring delivery of cells to the desired retinal location. This will be particularly important when attempting to either target or avoid placement under the macula, as might be envisioned in the setting of retinal degenerative diseases such as ARMD and retinitis pigmentosa, respectively. In addition, the inclusion of a polymer in the composite affords the opportunity of providing polymer-based extended drug delivery to the graft site [12].

Looking ahead, our data strongly suggest that polymer composite grafts will ultimately provide an improved method of RPC transplantation, as compared with single-cell suspensions, although this technology is not yet mature. As it evolves, polymer scaffolds may allow the delivery of larger, more flexible grafts that can be "scrolled up," drawn into a cannula, and extruded into the subretinal space through a small retinotomy, such as has previously been proposed for photoreceptor and retinal pigment epithelial grafts [31, 32]. The PLGA/PLLA polymer used in the present study, although providing an excellent substrate for cell attachment, does not possess the flexibility required for this delivery method. It may, however, be possible to develop a hybrid hydrogel, or other polymer, with the necessary properties. Similarly, the polymer we have used has a minimum thickness of approximately 150 μm, which is substantially thicker than the photoreceptor layer of the mouse retina. This physical constraint presents a substantial barrier to functional photoreceptor replacement in mouse models. We have therefore

begun studies in the pig, an animal model with an eye similar in size, structure, and function to that of humans. Demonstration of functional efficacy in such a model represents an important prerequisite to clinical application of polymer composite graft technology. We suggest that the use of a biodegradable polymer/progenitor cell composite graft offers the opportunity of achieving this goal and, potentially, a therapeutic option for degenerative diseases of the retina.

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Disclosures

The authors indicate no potential conflicts of interest.

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