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Engineering retinal progenitor cell and scrollable poly(glycerol-sebacate) composites for expansion and subretinal transplantation

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Abstract

Retinal degenerations cause permanent visual loss and affect millions world-wide. Presently, a novel treatment highlights the potential of using biodegradable polymer scaffolds to induce differentiation and deliver retinal progenitor cells for cell replacement therapy. In this study, we engineered and analyzed a micro-fabricated polymer, poly(glycerol sebacate) (PGS) scaffold, whose useful properties include biocompatibility, elasticity, porosity, and a microtopology conducive to mouse retinal progenitor cell (mRPC) differentiation. *In vitro* proliferation assays revealed that PGS held up to 86,610 (± 9993) mRPCs per square millimeter, which were retained through simulated transplantations. mRPCs adherent to PGS differentiated toward mature phenotypes as evidenced by changes in mRNA, protein levels, and enhanced sensitivity to glutamate. Transplanted composites demonstrated long-term mRPC survival and migrated cells exhibited mature marker expression in host retina. These results suggest that combining mRPCs with PGS scaffolds for subretinal transplantation is a practical strategy for advancing retinal tissue engineering as a restorative therapy.

Keywords

Biodegradation; Cell adhesion; Elastomer; Stem cell; Nerve tissue engineering; Ophthalmology

1. Introduction

Age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are diseases characterized by progressive deterioration of the retina, ultimately leading to clinically significant visual loss [1]. Present treatment strategies are designed to delay disease progression via gene therapy, growth factor treatment, and anti-angiogenic therapy. An emerging therapeutic paradigm to regenerate damaged retina is retinal progenitor cell (RPC) transplantation [1–3]. While the adult mammalian retina does not spontaneously regenerate, studies have shown that retinal tissue can be replaced and some degree of functional recovery regained following the delivery of RPCs to the subretinal space [2,3]. Most groups inject RPC suspensions directly into the retinal environment which causes massive new cell loss through efflux and death [2,3]. Research focused on improving RPC transplantation technology has involved developing a microscale, localizable vehicle for targeted progenitor cell delivery. Current retinal tissue engineering strategies use biocompatible polymer scaffolds (10–300 μm thick) to facilitate survival and integration of transplanted RPCs in host retinal tissue following transplantation [4–10].

Retinal tissue engineering is an invaluable tool for directing the fate of progenitor cells and for addressing the lack of available tissue for transplantation. Progenitor cells alone have limited capability of recreating complex tissues upon transplantation [4]. When cells are grown under the appropriate conditions on a three-dimensional scaffold, they become capable of developing mature genetic expression patterns and morphology [11,12]. Additionally, biodegradable polymers provide temporary scaffolding that is absorbed by the host resulting in *de novo* tissue [4,8].

A number of polymers have been shown to be non-cytotoxic in the eye, including the hydrogels, poly(caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), and poly(glycolic acid) (PGA) [13–15]. Previous studies using poly(L-lactic acid) and poly(lactic-co-glycolic acid) (PLLA/PLGA), poly(methyl methacrylate) (PMMA), and PCL have demonstrated that polymers enhance cell survival during transplantation by 14-fold over cell delivery via bolus injection [4]. These studies also demonstrated that the porosity of polymers enhances cell adhesion and that their microtopology can guide progenitor cells to mature toward functional phenotypes [5,7,8]. The above findings suggest that an ideal polymer for retinal tissue engineering should be less than 50 μm thick, display non-cytotoxicity, retain mechanical strength during transplantation, and biodegrade through hydrolysis within 6 months.

Here, we present a biocompatible, highly elastic and transplantable poly(glycerol sebacate) (PGS) scaffold. This micro-fabricated PGS scaffold is 45 μm thick with 50 μm diameter pores spaced 175 μm apart [9,16]. The porosity of PGS allows for efficient cell infiltration and retention during transplantation, as well as nutrient transport *in vivo*. Importantly, the elastomeric property of PGS allows the polymer (seeded with cells) to be scrolled and transplanted via syringe injection. Furthermore, with an *in vivo* degradation time of 1–4 months, PGS is the most rapidly degrading polymer that has been evaluated so far for retinal tissue engineering.

We have investigated the effect of PGS on mRPC differentiation, delivery, and subsequent migration into host retinal tissue. Proliferation and adhesion analyses demonstrated robust cell growth and survival following cell seeding of PGS scaffolds *in vitro*. mRPCs seeded on PGS differentiated toward mature retinal neurons as evidenced by changes in mRNA and protein levels of stemness and neuronal markers. In addition, glutamate induced calcium influxes were enhanced for mRPCs cultured on PGS for 7 days. *Ex vivo* studies demonstrated mRPC integration into retinal layers in normal and rhodopsin knockout (ρ -/-) retinal explant models. Finally, transplantation of mRPC-PGS composites into the subretinal space of C57bl/6 and ρ -/- mice for one month resulted in new cell migration into the host retina, differentiation, and long-term survival.

2. Methods and materials

2.1. Mouse progenitor cell isolation and culture

All experiments were performed according to the Schepens Eye Research Institute Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Isolation of mRPCs was performed as previously described [4]. Retinas were isolated from post-natal days 0–3 enhanced green fluorescent protein positive (GFP⁺) transgenic mice (C57BL/6 background). Pooled retinas were dissociated by mincing, and digested with 0.1% type 1 collagenase (Sigma–Aldrich; St. Louis, MO) for 20 min. The liberated mRPCs were passed through a 100 μ m mesh filter, centrifuged at 850 rpm for 3 min, re-suspended in culture medium Neurobasal (NB; Invitrogen–Gibco, Rockville, MD) containing 2 mM L-glutamine, 100 mg/ml penicillin–streptomycin, 20 ng/ml epidermal growth factor (EGF; Promega, Madison, WI) and neural supplement (B27; Invitrogen–Gibco) and plated into culture wells (Multiwell, Becton Dickinson Labware, Franklin Lakes, NJ). Cells were provided 2 ml of fresh culture medium on alternating days for 3 weeks until mRPCs were visible as expanding non-adherent spheres. mRPCs were passaged 1:3 every 7 days.

2.2. Polymer fabrication

2.2.1. Microfabrication of polydimethylsiloxane (PDMS)—All fabrication procedures were carried out in a class 10,000 clean room. An 80 μ m thick layer of SU8-2050 was spin-coated on a silicon wafer (4 inch diameter) following the manufacturer's instructions. The photoresist was patterned using a transparency mask (PageWorks, Cambridge, MA) with the ink-side down and developed using washes of propylene glycol monomethyl ether acetate and isopropanol. The patterned silicon wafer was prepared for PDMS replica molding by treating with a low surface energy release agent (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane. Briefly, two drops of (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane were applied to a glass slide, which was placed on the floor of a vacuum chamber containing the patterned silicon wafer. A vacuum was applied and the (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane vapor allowed to react with the wafer for at least 20 min. The PDMS negative mold was prepared from the patterned silicon wafer as described [9].

2.2.2. Fabrication of PGS scaffolds—Fabrication of the PGS scaffolds was also carried out in a class 10,000 clean room. The PDMS negative mold was oxidized by plasma treatment for 1 min to create a hydrophilic surface [8–10]. A 61.5% aqueous sucrose solution (0.2 mM filtered) was spin-coated at 3000 rpm for 30 s on the oxidized PDMS mold within 5 min of plasma treatment. The sucrose-coated PDMS was immediately baked at 135 °C in an oven for 10 min and then transferred to a 120 °C hotplate. Approximately 6.5 g of molten PGS (150 °C) were spin-coated at 3000 rpm for 30 s on the sucrose-coated PDMS molds. The PGS on the PDMS mold was cured at 120 °C under a vacuum of 15 mTorr for 48 h. Subsequently, the mold was submerged in ddH₂O for 16 days to loosen the PGS from the PDMS mold. The PGS was precut into pieces using a razorblade, and the pieces were gently peeled off the PDMS mold while submerged in ddH₂O using forceps. To examine the PGS scaffold by scanning electron microscope (SEM), the scaffold was coated with Au/Pd using a Hummer Sputter Coater, according to the manufacturer's instructions using an evaporator (Anatech Inc., Hayward, CA) [17].

2.3. Polymer preparation, cell seeding and culture

PGS scaffolds were cut with a sterile scalpel to 1 × 1 mm for proliferation, immunocytochemistry, and explants/transplants and to 2×2 mm for transplant simulation protocols. In our control analyses of mRPC proliferation, we seeded known numbers of cells into culture wells with a piece of PGS covering the well floor. The proliferation rates of mRPCs in culture wells alone or on PGS in identical wells showed no significant differences. Also, 1×1 mm sections of glass were cut from cover glass (VWR) for differentiation (Imaris) and calcium imaging controls and prepared in an identical manner to PGS for cell seeding. PGS and glass squares were incubated in 70% ethanol for 24 h and rinsed 3 times with Phosphate Buffered Saline (PBS) Solution. PGS scaffolds were placed into single wells of 12 well culture plates and incubated in 100 µg/ml mouse laminin (Sigma) in PBS for 1 h. Polymers were then rinsed 3 times with PBS and transferred to 0.4 µm pore culture well inserts (Falcon) in 12 well plates. Scaffolds were then submerged in 1 ml of NB and incubated for 1 h at 37 °C. Cultured GFP⁺ mRPCs were dissociated into single cell suspensions and seeded onto PGS membrane and glass. The total volume of NB in each well was brought to 2 ml with NB media, and mRPCs were allowed to proliferate on the polymer for 7 days at 37 °C.

2.4. Scanning electron microscopy

Prior to imaging, the cells were fixed and dehydrated. Each sample was rinsed twice in PBS and then soaked in a primary fixative of 3% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1 M sucrose for 72 h. The surfaces were subjected to two 5 min washes with a buffer containing 0.1 M sodium cacodylate and 0.1 M sucrose. The cells were then dehydrated by replacing the buffer with increasing concentrations of ethanol for 10 min each. The cells were dried, by replacing ethanol with hexamethyldisilazane (HMDS) (Polysciences) for 10 min, and subsequently air-dried for 30 min. As discussed previously, following mounting, samples were sputtercoated with a 15 nm layer of Au/Pd at a current of 20 mA and a pressure of 0.05 mbar for 45 s. SEM imaging was conducted on a FEI XL30 Sirion Scanning Electron Microscope at 5 kV.

2.5. Cell proliferation on PGS

Expansion of GFP⁺ mRPCs was analyzed on PGS. To establish a standard mRPC population curve, total mRPC GFP⁺ signals were detected from known cell number curves ($n = 5$) from 1×10^3 to 1.5×10^5 cells in a 96 well plate using a Tecan, Genios microplate reader. 1×1 mm PGS seeded with 2.5×10^5 mRPCs were cultured and imaged for 7 days. Total GFP⁺ emissions from mRPCs on each polymer were taken at days 1, 3, and 7 under identical conditions. The signals from mRPCs on PGS and standard population curve signals were then correlated to establish cell density on each day. After the initial seeding of cells, a Spot ISA-CE camera (Diagnostic Instruments, Sterling Heights, CA) attached to a Nikon Eclipse TE800 microscope was used to visualize cell proliferation across the surface and within the pores of PGS. The composites were also imaged at 10 $\times$ magnification on days 1, 3, and 7.

2.6. Immunofluorescence

After culturing mRPCs for 7 days, mRPC-PGS and mRPC-glass composites were rinsed 3 times with PBS (warmed to 37 °C) and fixed in 4% paraformaldehyde for 1 h. mRPCs to be analyzed for polymer-influenced differentiation were then processed for immunocytochemistry as described below. Explanted and transplanted tissue was then cryoprotected first in 10% sucrose for 12 h and then in 30% sucrose for 12 h. Cryoprotected composites were frozen in Optimal Cutting Temperature Compound (Sakura Finetek, Torrance, CA) at -20 °C and cut into 20 μ m sections using a Mino-tome Plus (Triangle Biomedical Sciences, Durham, NC). All samples were then rinsed 3 times for 10 min each in PBS, blocked, and permeabilized in PBS containing 10% goat serum, 1% BSA, and 0.1% Triton-X for 2 h. Samples used to compare differences (Imaris, Bitplane, Inc., Saint Paul, MN) between PGS and glass influenced mRPC genetic expression were incubated with the primary antibodies:paired box gene 6 (Pax6) (Hybridoma Bank, Iowa City, IA) 1:20, Hairy and enhancer of split 1 (Hes1) (Chemicon) 1:200, Ki67 (Sigma, St. Louis, MO) 1:100, nestin (BD Biosciences, San Jose, CA) 1:200, SRY(sex determining region Y)-box2 (Sox2) (Chemicon, Billerica, CA) 1:200 and glial fibrillary acidic protein (GFAP) (Zymed, San Francisco, CA) (1:200). PGS-explant and transplant samples were incubated with the primary antibodies:GFAP 1:200, cone-rod homeobox (crx) (Santa Cruz, Santa Cruz, CA) 1:100, Recoverin (Abcam, Cambridge, MA) 1:200, neural-filament-200 (nf-200) (Sigma) 1:400, neuronal nuclei (NeuN) (Invitrogen, Carlsbad, CA) 1:100, rhodopsin (Sigma) 1:100, nestin 1:200, Protein kinase C (PKC) (Sigma) 1:200 in blocking buffer for 12 h at 4 °C. Samples were then rinsed 3 times for 10 min each in PBS and incubated with a rhodamine-conjugated secondary antibody 1:800 (Zymed) and Topro-3 (Invitrogen) nuclear stain for 2 h at room temperature. Finally, samples were rinsed 3 $\times$ 10 min in PBS and sealed in mounting medium (Vector Laboratories, Burlingame, CA) for imaging using a Leica TCS SP2 confocal microscope. GFP⁺ mRPCs were analyzed for the expression of markers labeled by immunocytochemistry between PGS and glass samples using the colocalization feature of the Imaris software (Bitplane Inc.). This software quantified the degree of colocalization between markers (in this case, between GFP and a certain marker for differentiation) for a certain threshold, which was held constant for both the glass and PGS samples [18,19].

2.7. Real-time quantitative RT-PCR (qPCR)

Total RNA was extracted from day 7 cell cultures on the PGS (RNeasy Mini kit Qiagen, CA) followed by column treatment with DNase I (Qiagen, CA). Reverse transcription was performed with Omniscriptase Reverse Transcriptase (Qiagen, CA) and random primers (Sigma) (Table 1). Real-time quantitative PCR was performed with 7500 real-time PCR system (Applied Biosystems, Irvine, CA) at 40 cycles with 100 ng of starting cDNA. The primers used in this study are shown in Table 1. Power SYBR green was used for amplification and data analyzed by delta CT method, SDS program version 1.4 (Applied Biosystems, Irvine, CA). RNA was quantified with the delta CT method and normalized to β -Actin as an endogenous control. Each reaction was performed in triplicate.

2.8. Calcium imaging

Substrate induced differences in calcium dynamics were analyzed between mRPCs cultured on PGS or glass for one week in modified Neurobasal. For Ca^{2+} imaging, cells were transferred to 35 mm glass bottom Petri dishes (MatTek, Ashland, MA) and allowed to adhere in Neurobasal at 37 °C for 1 h. mRPCs were then rinsed with Ringer's solution maintained at 37 °C containing in: (mM) NaCl 119, KCl 4.16, CaCl 2.5, MgCl 0.3, MgSO 0.4, Na_2HPO_4 0.5, NaH_2PO_4 0.45, HEPES 20, Glucose 19 at pH 7.4. Cells were then incubated in Ringer's solution containing 0.5 μM fura-2 tetra-acetoxymethyl ester (Fura-2) (Molecular Probes), 10% pluronic F127 (Sigma), and 250 μM sulfinpyrazone (Sigma) for 40 min at 22 °C. Fura-2 was excited by alternating 340 and 380 nm light with the use of filter changer, under the control of InCytIM-2 software (Intracellular Imaging Corp., Cincinnati, OH) and paired to a Nikon Eclipse T5 100 Microscope. A new ratioed (340/380) image was obtained every 0.35 (s) as a measure of Ca^{2+} concentration. Background intensity was zero. A bolus injection brought the stimulant concentration in the cell bath to either 1 mM glutamate (Sigma) or 1 mM (*N*-methyl-D-aspartic acid) NMDA plus the co-stimulator 1 mM glycine (Sigma).

2.9. Retinal transplantation culture ex vivo

The *ex vivo* retinal culture was prepared as previously described [20]. Briefly, C57bl/6 and rhodopsin knockout ($\text{rho}^{-/-}$) mice ($n = 5$ each) were euthanized and their eyes enucleated immediately and placed in ice cold PBS. The anterior portion of each eye was removed along with the vitreous. Four radial cuts were made into the posterior eyecup and each quadrant flattened sclera side down. The flattened eyecup was then cut into four separate pieces ($\sim 2.0 \times 2.0$ mm) and the neural retina transferred to a 0.4 μm culture well insert, ganglion side down, with the sclera, choroid plexus and retinal pigment epithelium removed. The 0.4 μm pores in the culture well inserts allowed for the passage of nutrients between the media each well of 6-well plates and the retinal explants on the upper surface of the insert. NB (2 ml) was added to each culture well. A 7-day cultured mRPC-PGS (1 $\times$ 1 mm) composite was placed with sterile forceps onto each retinal explant (Fig. 1). mRPC seeded PGS composites were added to both C57bl/6 ($n = 5$) and $\text{rho}^{-/-}$ explants ($n = 5$) and cultured for one week in NB at 37 °C.

2.10. In vivo subretinal transplantation surgery

Transplantation surgeries were performed as previously described [4,8]. Briefly, PGS scaffolds with adherent RPCs were cut into 1.0×0.5 mm sections using a sterile scalpel in preparation for transplantation. Mice were placed under general anesthesia with an intraperitoneal injection of ketamine (5 mg/kg) and xylazine (10 mg/kg) and the pupil dilated with 1% tropicamide, topically applied. Proparacaine (Akorn), a local anesthetic, was applied to the eye. The temperature of mice was maintained at 37 °C using a heating blanket and heat lamp during surgery. Silk thread (8-0) was used to suture the eyelid open and the eye was stabilized using a single 11-0 conjunctival suture. An incision (1.0–2.0 mm) was made in the lateral posterior sclera using a Sharp point 5.0 mm blade scalpel (Fine Science Tools, Reading, PA) PGS–mRPC composites were placed into a 0.5 mm I.D. glass or polyethylene tube for injection via a syringe fit with a 6 cm long, 0.5 mm O.D steel plunger. PGS–mRPC composites were then injected through the sclerotomy into the subretinal space between the retina and RPE. A single eye from each C57BL/6 wild-type mouse ($n = 5$) and rho–/– ($n = 5$) received a sub-retinal transplant. The scleral incision was closed with an 11-0 nylon suture, and all other sutures were removed. Additional proparacaine was applied, and the mice were allowed to recover. Transplants remained in the subretinal space for one month.

2.10.1. Histologic analysis of transplanted tissue—C57BL/6 mice that received composite grafts were sacrificed after 4 weeks. Engrafted eyes were enucleated, immersion fixed in 4% paraformaldehyde, rinsed 3 times in PBS and cryoprotected in 10%, then 30% sucrose for 12 h each at 4 °C. Eyes were then placed in a cryomold containing optimum cutting temperature (O.C.T.) (ProSciTech) and then frozen on dry ice, and cryosectioned at 18 μ m.

3. Results

3.1. Polymer preparation, cell seeding and culture

As PGS scaffolds were to be eventually scrolled and inserted into a medium gauge (0.5 mm inner diameter) syringe for subretinal injection, preservation of the elastic properties of PGS during the preparation necessary for cell culture and eventual transplantation was one of the primary objectives in the evaluation of this scaffold. The *in vitro* release of a non-seeded PGS control has been included (Supplemental Movie 1, scale, 1 mm). 1×1 mm PGS scaffolds, sterilized for 6–8 h in 70% ethanol and coated with 100 μ g/ml of laminin, maintained sufficient elasticity to be scrolled and then spontaneously unroll in solution (Fig. 2a and d). Each scaffold was then seeded with 2.5×10^5 mRPCs isolated from GFP⁺ transgenic mice. At 24 h post-seeding, approximately 4% of the cells (9077 ± 4748 cells; error = s.e.m.) remained attached (Fig. 2b). This percentage of cell attachment can be attributed to the flow of mRPCs in suspension around the polymer toward corner regions of the porous culture well insert. However, by day 3, adherent mRPC numbers had more than doubled to 20,003 ($\pm 10,223$) and by day 7 the population had significantly expanded (*t*-tests: $p = 0.002$ between days 1 and 7; $p = 0.004$ between days 3 and 7) to 86,610 (± 9933) (Fig. 2c and e). The appearance of mRPC colony structures, neurospheres, at later time points indicated normal cell proliferation patterns (Fig. 2c). Fluorescent cells were visible on both

surfaces of the PGS scaffolds as well as within pores (Fig. 2f). The PGS scaffold also proved to have superior cell adhesion properties when compared to laminin coated (100 µg/ml) glass (Fig. 2g). Under identical seeding and culture conditions, 2×2 mm PGS scaffolds retained a significantly higher number of cells than glass after both were transferred with forceps between two 35 mm medium filled culture wells to simulate initial subretinal transplantation shearing forces. Following transfer, mRPC-PGS ($n = 3$) and mRPC-glass ($n = 3$) composites retained 229,992 ($\pm 68,021$) and 15,596 ($\pm 12,418$) cells, respectively ($p = 0.03$). The superior cell adhesive property of PGS was predicted based on our previous analysis of optimal polymer characteristics for this application [5].

3.2. Scanning electron microscopy (SEM) of mRPC seeded scaffolds

The PGS scaffold maintained a thickness of 45 µm with 50 µm diameter pores both before and after seeding and cell culture with mRPCs for one week. No deformation or loss of scaffold integrity was observed at the SEM level (Fig. 3a and d). The morphology of mRPCs attached to PGS at day 7 appeared to include flattened radial phenotypes, in direct contact with the polymer, bipolar and spheroid bodies bordering and within neurospheres (Fig. 3b,c,e, and f). Imaged from above, flattened, bipolar and spheroid mRPCs could be seen within PGS pores, with many extending cytoplasmic processes between cells and between cell and polymer (Fig. 3b and c). Cross-sections of pores showed cells attached to the inner walls and filling the pore in approximately four-cell layers high (Fig. 3e and f).

3.3. Quantitative immunofluorescence analysis of mRPCs on PGS and glass

GFP⁺ mRPCs cultured on either PGS or glass for seven days were processed for indirect immunofluorescence analysis to examine the expression profiles of several stemness and differentiation markers. Antibodies for these markers were visualized by rhodamine-conjugated secondary antibodies. Confocal microscope images were then analyzed using the Imaris software to quantitatively determine the fraction of GFP⁺ mRPCs positive for rhodamine fluorescence (see Methods). Three z-stacks, each containing 50 optical slices, were analyzed per marker. Percentages of cells expressing selected markers on glass and PGS (in this order) were as follows: Pax6 = 41.92 (± 5.16), 15.47 (± 6.42); Hes1 = 66.86 (± 6.03), 27.06 (± 1.74); Ki67 = 24.80 (± 3.61), 30.76 (± 1.79); nestin = 41.32 (± 16.48), 17.48 (± 1.00); Sox2 = 47.95 (± 1.81), 1.58 (± 0.30); and GFAP = 32.41 (± 16.17), 8.46 (± 0.56). The canonical markers of stemness or undifferentiation for this population of mRPCs include the eye-fate nuclear transcription factors Pax6 and Sox2, a repressor of cell differentiation, Hes1, and the intermediate filament protein, nestin. mRPCs cultured on PGS expressed lower levels of these stemness proteins as compared to glass, indicating a potential trend toward maturation (Fig. 4a–g; GFP is shown in green and antibody staining in red in Fig. 4b – g). Expression of the active cell-cycle marker Ki67 appeared similar for both populations suggesting that a number of mRPCs continue to proliferate on both substrates.

3.4. RT-qPCR analysis of mRPCs grown on PGS and glass

The expression profiles of the markers discussed above were confirmed at the mRNA level by reverse transcription-qPCR analysis (Fig. 4h). Of the six mRNA species examined, four showed decreased expression in association with growth on PGS as compared to glass.

These were Pax6, Hes1, Sox2 and GFAP. Ki-67 and nestin mRNA levels, on the other hand, were higher in PGS cultured cells. These RT-qPCR analysis results paralleled those of the immunofluorescence analysis with the exception of nestin, which showed an increase in mRNA signal compared to lower detected protein signal. This may be a reflection of decreased translation and consequent stockpiling of mRNA [21].

3.5. Calcium imaging

Intracellular calcium dynamics in response to transmitter stimulation were analyzed for mRPCs cultured on either glass or PGS for seven days (Fig. 5). Individual cells were analyzed for changes in Fura-2 fluorescence as an indicator of changes in intracellular calcium levels (see Fig. 5c for an example of fluorescence before and after stimulation). The percentage of mRPCs responsive to 1 mM glutamate was derived by dividing the number of responsive cells by the total number of cells per condition. This analysis revealed that 96% of the total number of cells responded on PGS while 92% responded on glass (Fig. 5a and b). Interestingly, the maximum calcium influx in response to glutamate was significantly higher (*t*-test, *p* = 0.001) on PGS (max avg: 641.16 nM; 39.78 s.e.m.; *n* = 25) than on glass (max avg: 458.92 nM; 37.42 s.e.m.; *n* = 25) (Fig. 5d). Responses to 1 mM NMDA with the co-stimulator 1 mM glycine were similar between groups. Responding, Fura-2 loaded, mRPCs returned to baseline with similar time courses in both PGS and glass samples.

3.6. Retinal explant, mRPC migration and differentiation

PGS-mRPC composites cultured for one week on C57bl/6 and rho^{-/-} retinal explants allowed for mRPC migration into retinal layers and expression of location and fate-specific markers (Fig. 6). GFP⁺ mRPC migration into C57bl/6 and rho^{-/-} explants resulted in high levels of migration into the inner nuclear (INL) and ganglion cell layers (GCL) (Fig. 6a and e). Analysis of 18-μm thick cryosections of C57bl/6 and rho^{-/-} explants showed an average of 175 (±57) and 94 (±33) migrated mRPCs per section, respectively (*n* = 5 sections each; Fig. 7f). A total of ~ 11,900 and 6400 mRPCs had migrated into the C57bl/6 and rho^{-/-} explants underlying the 1×1 mm PGS composites, respectively. New cell migration appeared dense in both nuclear and plexiform layers of the C57bl/6 explants. Immunofluorescence analysis showed that GFP⁺ cells that had migrated from PGS composites into C57bl/6 retina expressed the intermediate filament protein, nf-200, in all retinal layers, nestin and NeuN (neuronal nuclei) in the outer nuclear layer (ONL), PKC in the INL region, and GFAP in the GCL region (Fig. 6a–e; GFP in green and antibody staining in red). In rho^{-/-} explants, cells still attached to the PGS composite expressed NeuN, cells that had migrated into the INL expressed crx, nestin in the inner plexiform layer (IPL) region, and GFAP in the GCL region (Fig. 6f – i). Reduced cell migration was observed in rho^{-/-} explants cultured with PGS-mRPC composites. A significant difference between C57bl/6 and rho^{-/-} retina is the degenerated ONL that typifies rho^{-/-} retina. The rho^{-/-} retinal phenotype is well characterized and retinal remodeling including glial hypertrophy and scarring occurs during loss of photoreceptor outer segments [22]. It has been established that stem cell transplantation into the subretinal space of mice with progressed retinal degeneration results in inhibited migration of new cells into host retina due to gliosis and extracellular matrix deposition [23].

3.7. Subretinal transplantation, mRPC migration and differentiation

Scrolled PGS–mRPC composites were transplanted via transscleral injection into the subretinal spaces of C57bl/6 and rho^{-/-} mice ($n = 5$ each) and unrolled to present their cell cargo to the outer segment layer. The *in vitro* release of a PGS–mRPC control has been included (Supplemental Movie 2, scale, 1 mm). In our disease model, rho^{-/-} mice, the degeneration of photoreceptor outer segments produced a retinal detachment into which composites were delivered. In control, wild-type mice, a localized retinal detachment was created in the region of composite transplantation, which produced a mild disorganization of outer segments. The composites remained *in vivo* for thirty days. At the time of analysis, no PGS was found remaining in the subretinal space and both rhodopsin knockout and wild-type retinal laminar organization were preserved. PGS delivered mRPCs migrated into host retinal tissue. Analysis of 18 μ m sections of both C57bl/6 and rho^{-/-} retina revealed an average of 28 (± 8) and 15 (± 4) migrated mRPCs, respectively ($n = 5$ sections each; Fig. 7g). Based on average migration areas of 550 $\times$ 250 μ m underlying the delivered PGS scaffolds, a total of approximately 672 and 360 mRPCs were estimated to have migrated into C57bl/6 and rho^{-/-} transplants, respectively. A subpopulation of PGS delivered mRPCs migrated into the inner plexiform layer of C57bl/6 retina and labeled positively for GFAP (Fig. 7a; arrows). Small diameter mRPCs migrated into the ONL and labeled positively for crx, NeuN, and rhodopsin (Fig. 7b – d). In the ONL migrated cells labeled positively for PKC (Fig. 7e). The data indicate that migrated mRPCs were neural (NeuN) and expressed location appropriate markers toward photoreceptor (crx, rhodopsin), and bipolar (PKC) fates.

4. Discussion

In this work, we demonstrate that the elastic and cell-compatible PGS scaffold is well suited for initial mRPC differentiation *in vitro* and subsequent *in vivo* delivery to the subretinal space of mouse. The mechanical properties of the PGS–mRPC composites facilitate scrolled injection into the subretinal space, thereby protecting cells from shearing forces and reducing trauma to ocular tissue during transplantation. This is the first use of a scrollable PGS scaffold for the delivery of mRPCs and serves to further advance efforts toward a practical cell replacement therapy for damaged retinal tissue. Following transplantation, PGS allows for the passage of nutrients and cells through its 50 μ m diameter pores [9]. In addition, the PGS gradually surface biodegrades through hydrolysis, reducing the chance of negatively affecting the pH of the subretinal microenvironment, as is common with bulk-degrading polymers like PLGA [9,15].

In our comparison of mRPC protein expression patterns on PGS and glass, we discovered that PGS promoted differentiation, a conclusion confirmed by RT-qPCR analysis (Fig. 3h). The microenvironmental cues potentially acting to influence mRPC expression behavior are worth considering here. First, to enhance cell adhesion, PGS is coated with laminin (100 μ g/ml), which is known to influence differentiation of progenitor cells toward mature retinal phenotypes [24,25]. Secondly, nanoscale surface variations (nanochannels and projections) present on the PGS surface, which are the result of the fabrication process, serve as topographic cues (Fig. 3a and d). These incident variations guide mRPC adherence, process

extension, and morphologic adaptations (Fig. 3c and f). A number of mRPCs in contact with PGS adhere and respond to the surface by developing a flattened morphology and extending radial processes. This observed cell behavior is supported by earlier findings describing the tendency of polymer topology to direct progenitor cell morphologic adaptations and subsequent changes in protein expression levels [26,27–30]. Surfaces with topographic cues that are similar to endogenous extracellular matrices can direct cell morphogenesis via cell-surface contact [8,29]. Topographic cues in previous retinal tissue engineering studies with mRPCs have included microscale pores, vertical nanowires, as well as nanoscale smooth and rough surfaces (4–10). Cellular responses to either structural or mechanical features of these environmental cues lead to mRPC differentiation toward mature retinal neural phenotypes (6,8). Polymer topology influenced differentiation is predicted to progress via cell binding to nano and microscale features followed by cell surface receptors acting as mechano-chemical transducers, activating signal transduction pathways and ultimately modulating gene expression and protein expression [30].

The PGS microenvironment also affected the response of mRPCs to glutamate (Fig. 5). RPCs express ionic glutamate receptors and functional changes during development have been shown to involve modification of subunit composition and increased calcium currents [31,32]. Photoreceptor, bipolar and ganglion cells express glutamate receptors. As 96% of PGS-cultured mRPCs responded to glutamate, these polymer-composite cells possess potential to function as players in the host retinal glutamate signaling pathways [33]. The mRPCs used for this study were isolated from GFP⁺ C57BL/6 mice at post-natal day 0, the period of retinal genesis shown to produce the highest percentage of rods [34–36]. While transplanted mRPCs expressed a range of mature markers, focused rod differentiation would be optimal. In the future, to improve specific differentiation of mRPCs in host retina toward a photoreceptor fate, it may be useful to drive cells to exit cell cycle *in vitro* prior to transplantation. Additionally, exposing post-mitotic mRPCs to exogenous factors such as taurine and retinoic acid may, in conjunction with PGS, drive mRPCs to express rhodopsin and other markers of photoreceptor fate [35,37].

In recent studies using subretinal bolus injections to deliver progenitor cells, low percentages of cells (<0.5%) were shown to migrate into retina [3,38]. By using PGS as a cell delivery vehicle, we demonstrate that at one week in C57bl/6 and rho^{-/-} explants, 13% and 7% of transplanted cells migrate into retina, respectively. Four weeks following subretinal transplantation of PGS–mRPC composites, *in vivo*, resulted in 1.5% of cells migrating into C57bl/6 retina and 0.8% into rho^{-/-} mouse retina. While these observations are encouraging, it is important to continue to evaluate and optimize surgical handling of composites and preparation of the retinal environment. Further studies are also necessary to evaluate polymer induced mRPC fate specification and terminal differentiation following transplantation.

5. Conclusions

We conclude that mRPCs can be combined with PGS scaffolds to form retinal tissue equivalents in culture. PGS demonstrates superior mRPC compatibility *in vitro* and influences trends toward differentiation. In our explant and transplant models, high numbers

of mRPCs migrated into retinal tissue. This paradigm offers a mechanism for localized delivery of new cells to areas of damaged retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Figures with essential colour discrimination. Parts of the majority of figures in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2009.02.046](https://doi.org/10.1016/j.biomaterials.2009.02.046).

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biomaterials.2009.02.046](https://doi.org/10.1016/j.biomaterials.2009.02.046).

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Fig. 1.

Retinal transplantation *ex vivo* method. a) GFP + mRPCs are seeded and allowed to proliferate for one week on PGS in a culture insert containing 0.4 μm pores which provides a restricted surface area and allows for the exchange of nutrients from culture media contained in the surrounding culture well. b) At day 7, freshly isolated retina are explanted onto a culture well insert with ganglion cell layer (GCL) at bottom, inner nuclear layer (INL) center and outer nuclear layer (ONL) at top. PGS-mRPC composites are then transferred to the ONL surface and allowed to culture and migrate into retinal lamina for 7 days.

Fig. 2.

Proliferation and adherence of mRPCs cultured on PGS. a) Elastic and porous PGS scrolled within a 1 mm inner diameter glass needle and d) injected and unscrolled (scale, 1 mm). b) Day 1 post-GFP⁺ mRPC seeded PGS shown left under white light and right under fluorescent illumination. c) GFP⁺ mRPCs fully cover PGS with proliferative neurospheres (arrows) by day 7 (scale b,c, 100 μ m). e) mRPC numbers on 1 $\times$ 1 mm PGS sections increased from 9077 (± 4748), 20,003 ($\pm 10,223$) and 86,610 (± 9933) at days 1, 3 and 7 respectively. (days 1–7, $*p = .002$, days 3–7, $*p = .004$) f) 40 $\times$ section of GFP mRPCs

adherent to PGS (pseudo color blue) on upper and lower surfaces and through pores (scale, 50 μm). g) Following identical treatment and mRPC seeding density (2.5×10^5), 2×2 mm PGS retained a significantly higher number of cells than glass after 7 days in culture transplantation simulation 229,992 ($\pm 68,022$) and 15,596 ($\pm 12,418$), respectively (* $p=0.03$). Error bars, s.e.m.

Fig. 3.

SEM of PGS topology and mRPC adhesion. a) Top view of PGS 45 μm thick PGS scaffold with 50 μm diameter pores spaced 175 μm apart. b) Top view of PGS seeded with mRPCs after 7 days of proliferation surrounding pore (circle). c) Magnification of b showing individual mRPCs with flattened radial and bipolar morphology. d) Side view of PGS scaffold showing the cone-like pore formation on the upper surface and 45 μm thickness. e) Adhesion of neurospheres to the PGS surface, along the sidewalls, and within individual pores (circle). f) Magnification of e showing spheroid mRPC infiltration into an individual pore.

Fig. 4.

Immunocytochemical and qPCR analysis of PGS influenced expression levels. To evaluate the influence of the PGS microenvironment on cell differentiation, intensity of immunocytochemical labeling was compared between mRPCs cultured on either 1 × 1 mm PGS or glass for 7 days. a) Marker expression indicating undifferentiated mRPCs including Pax6, Hes1, nestin, and Sox2 was decreased in cells cultured on PGS. Elevated GFAP expression may indicate a higher percentage of mRPCs of glial fate on glass than on PGS. b,e) GFP⁺ mRPCs expressing c,f) nestin were analyzed in overlaid images d,g) for percent

expression between glass (b–d) and PGS (e–g) samples. Cells cultured on PGS expressed lower levels of nestin. h) Results from quantitative PCR analysis showed that message expression correlated well with protein data, the exception being an increase in the level of nestin message in response to growth on PGS (b–g) (green = GFP, red = rhodamine labeled protein).

Fig. 5. mRPCs respond to glutamate, NMDA + glycine with Ca^{2+} influxes. a,b) Averaged responses of Fura-2 loaded PGS-mRPCs ($n = 25$) demonstrate a 40% greater peak intracellular Ca^{2+} influx above glass-mRPCs ($n = 25$) in response to 1 mM glutamate. c) Pseudo color images illustrating 340:380 ratio levels of Fura-2 loaded mRPCs before (left) and at the peak of glutamate stimulation (right) (scale, 50 μm). d) Responses of mRPCs stimulated with 1 mM glutamate, 1 mM NMDA with 1 mM glycine. Averaged glutamate responses by mRPCs on PGS (max avg: 641.16 nM, 39.78 s.e.m., $n = 25$) were significantly higher than those on glass (max avg: 458.92 nM, 37.42 s.e.m., $n = 25$, $**p = 0.001$) Responses to NMDA with glycine were not significantly different between groups.

Fig. 6.

PGS scaffold delivery of GFP⁺ mRPCs to C57bl/6 and rho^{-/-} mouse retinal explants. Scaffolds were seeded with $\sim 2.5 \times 10^5$ at day P0 with GFP⁺ mRPCs (green) and allowed to proliferate *in vitro* for 7 days. a-e) C57bl/6 retina. a) GFP⁺ mRPCs co-labeled (yellow) for nf-200 (arrows), delivered on PGS aligned with the ONL from which cells extended processes and migrated into each retinal layer. b) PGS delivered mRPCs label for nestin in the ONL, c) NeuN (neuronal nuclei) in the ONL, d) PKC in the INL region, and e) GFAP in the GCL region. Antibody staining is shown in red. f-j) rho^{-/-} retina. The ONL has degenerated in these rho^{-/-} retina and PGS with attached layer of mRPCs aligned with the INL. mRPCs migrated into each of the remaining inner nuclear, inner plexiform and ganglion cell layer. f) GFP⁺ mRPCs that reached the GCL layer expressed GFAP (arrows). g) mRPCs still attached to the PGS scaffold labeled for NeuN, h) cells that reached the INL expressed crx, i) nestin in the IPL region, and j) GFAP in the GCL region (green = GFP, red = rhodamine labeled marker, blue = Topro-3 nuclei label) scale, 25 μ m.

Fig. 7.

GFP⁺ mRPC migration and differentiation in C57bl/6 retina 30 days following sub-retinal transplantation. a) PGS delivered GFP⁺ mRPCs (green) migrate into the inner plexiform layer and co-label for GFAP (red). Inset a) shows a PGS-RPC composite scrolled and loaded (left) into a 1 mm I.D glass tube and ejected (right) unrolled to present its cell cargo (scale, 1 mm). Small mRPCs migrated into the ONL label for b) *crx*, c) in NeuN, and d) rhodopsin. In the inner nuclear layer migrated cells label for PKC (scale, 50 μ m). f) At day 7, the number of mRPCs migrated into 18 μ m thick C57bl/6 and rho^{-/-} retinal explant sections were 175 $\pm$ 57 and 94 $\pm$ 33, respectively. g) The number of mRPCs in each 18 μ m section of C57bl/6 and rho^{-/-} retina at 30 days post-transplantation was 28 $\pm$ 8 and 15 $\pm$ 4, respectively (green = GFP, red = rhodamine labeled protein, blue = Topro-3 nuclei label).

Table 1

List of primers and product size for qRT-PCR.

Gene	Primer sequence (5'-3')	Product size (bp)
Nestin	F: AACTGGCACACCTCAAGATGT	235
	R: TCAAGGGTATTAGGCAAGGGG	
Sox2	F: CACAACCTCGGAGATCAGCAA	190
	R: CTCCGGGAAGCGTGACTTA	
Pax6	F: AGTGAATGGGCGGAGTTATG	132
	R: ACTTGGACGGGAAGTACAC	
Hes1	F: CCCACCTCTCTTCTGACG	185
	R: AGGCGCAATCCAATATGAAC	
Ki-67	F: CAGTACTCGGAATGCAGCAA	170
	R: CAGTCTTCAGGGGCTCTGTC	
GFAP	F: AGAAAACCGCATCACCATTC	184
	R: TCACATCACCACGTCCTTGT	