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Controlled microenvironments to evaluate chemotactic properties of cultured Müller glia

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ABSTRACT

Emerging therapies have begun to evaluate the abilities of Müller glial cells (MGCs) to protect and/or regenerate neurons following retina injury. The migration of donor cells is central to many reparative strategies, where cells must achieve appropriate positioning to facilitate localized repair. Although chemical cues have been implicated in the MGC migratory responses of numerous retinopathies, MGC-based therapies have yet to explore the extent to which external biochemical stimuli can direct MGC behavior. The current study uses a microfluidics-based assay to evaluate the migration of cultured rMC-1 cells (as model MGC) in response to quantitatively-controlled microenvironments of signaling factors implicated in retinal regeneration: basic Fibroblast Growth factor (bFGF or FGF2); Fibroblast Growth factor 8 (FGF8); Vascular Endothelial Growth Factor (VEGF); and Epidermal Growth Factor (EGF). Findings indicate that rMC-1 cells exhibited minimal motility in response to FGF2, FGF8 and VEGF, but highly-directional migration in response to EGF. Further, the responses were blocked by inhibitors of EGF-R and of the MAPK signaling pathway. Significantly, microfluidics data demonstrate that changes in the EGF gradient (i.e. change in EGF concentration over distance) resulted in the directional chemotactic migration of the cells. By contrast, small increases in EGF concentration, alone, resulted in non-directional cell motility, or chemokinesis. This microfluidics-enhanced approach, incorporating the ability both to modulate and asses the responses of motile donor cells to a range of potential chemotactic stimuli, can be applied to potential donor cell populations obtained directly from human specimens, and readily expanded to incorporate drug-eluting biomaterials and combinations of desired ligands.

1. Introduction

Retinal dysfunction and disease are leading causes of progressive and irreversible vision loss worldwide (Quigley and Broman, 2006), (Wong et al., 2014). Emerging studies have begun to evaluate the abilities of Müller glial cells (MGCs) to generate new neurons following retinal injury. MGC-mediated regeneration or neural cell delivery may thereby complement cell-replacement strategies using retinal progenitors and stem-like cells (Zhao et al., 2017; Santos-Ferreira et al., 2016; Klassen, 2016). MGCs provide primary structural and trophic support for retinal neurons (Bringmann et al., 2006; Willbold and Layer, 1998) and are known to react acutely to changes in the retinal microenvironment via migration, proliferation and expression of neurotropic factors (Lenkowski and Raymond, 2014; Tackenberg et al., 2009; Taylor et al., 2015). Developing therapies have embedded MGCs within specialized biomaterial grafts (Becker et al., 2016; Bull et al., 2008; Lorber et al., 2015) and re-programmed MGCs into precursors of retinal neurons (Sanges et al., 2016; Das et al., 2006; Joly et al., 2011; Ooto et al., 2004; Singhal et al., 2012) to harness MGC neuroprotective responses that prevent neuronal death and isolate injured or diseased cells (Dreyfus et al., 1998; Machalinska et al., 2013). The migration of MGC donors, thereby, becomes central to these reparative strategies, as cells must achieve appropriate positioning to facilitate localized repair (Santos-Ferreira et al., 2016; Thakur et al., 2018).

Finely-tuned migration of donor cells undoubtedly includes chemotactic processes across concentration gradient fields of biochemical compounds (Reviewed in (Shellard and Mayor, 2016)). Chemical cues from the retinal microenvironment have been implicated in MGC migratory responses of numerous retinopathies (Hollborn et al., 2005; Lorenc et al., 2015; Luo et al., 2016), as MGCs have been reported in sub-retinal spaces adjacent to sites in need of repair (Tackenberg et al., 2009; Humphrey et al., 1993), (Lee et al., 2008) and shown to express

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putative receptors for many of the factors secreted by degenerating photoreceptors (Bringmann et al., 2006; Dreyfus et al., 1998). In addition, numerous projects have reported MGC migration in response to extrinsic growth factors, including EGF (Meuillet et al., 1996a), HB-EGF (Hollborn et al., 2005; Hu et al., 2014; Inoue et al., 2013), FGF (Romo et al., 2011), TGFb (Luo et al., 2016), IGF-1 (Lorenc et al., 2015) and VEGF (Gaddini et al., 2016), as well as lipid receptors LRPI (Barcelona et al., 2013) and S1PR1 (Esche et al., 2010). Surprisingly, many potentially MGC-reliant therapies have yet to explore the extent to which external biochemical stimuli can direct or regulate MGC migration to promote retinal repair.

Microfluidic systems provide opportunities for sensitive assessment of cell responses to sophisticated chemical gradients with and without extracellular matrixes (Reviewed in (Sackmann et al., 2014; Wu et al., 2013; Ricoult et al., 2015)). Significantly, microfluidics enables precise control of concentration gradients that fundamentally drive chemotactic, or directional, migratory processes. This is particularly advantageous for modeling of retinal gradients, which are likely to be extremely steep and are developed over short anatomical distances (Goodhill, 2016). The current study uses a microfluidic assay, called the μLane, to evaluate MGC migratory behaviors in response to quantitatively-controlled environments at the retinal microscale (Beck et al., 2016; Dudu et al., 2012; McCutcheon et al., 2017; Rico-Varela et al., 2015; Unachukwu et al., 2013, 2016). This work examines the migration of a model MGC cell population in response to extrinsic signaling factors implicated in retinal regeneration: basic Fibroblast Growth factor (bFGF or FGF2) (Taylor et al., 2015; Meuillet et al., 1996b); Fibroblast Growth factor 8 (FGF8) (Fischer et al., 2009; Wan and Goldman, 2017); Vascular Endothelial Growth Factor (VEGF) (Gaddini et al., 2016; Saint-Geniez et al., 2008); and Epidermal Growth Factor (EGF) (Dreyfus et al., 1998; Hu et al., 2014). The rMC-1 cell line was chosen to initially define the experimental system prior to investigations using primary Müller glia because the line is genetically well-characterized and expresses induced/basal markers of primary glia and receptors for critical signaling proteins (Jiang et al., 2006, 2014; Muto et al., 2014; Yu et al., 2009). Further, rMC-1 cells have been widely-used with animal studies in the development of retinal therapies (Qiu et al., 2017; Xu et al., 2015; Cui et al., 2012).

Our findings indicate that rMC-1 cells exhibited minimal motility in response to FGF2, FGF8 and VEGF, but highly-directional migration in response to EGF. Further, the responses were blocked by inhibitors of EGF-R and of the MAPK signaling pathway. Interestingly, the chemotactic responses appeared to be preferentially activated by high EGF gradient fields, as migration in response to, both, moderate and lower EGF gradients elicited directionality and cell displacements comparable to control conditions (i.e. no EGF gradients). These results highlight the extent to which microfluidics-enhanced study can advance development of reparative therapies by manipulating cell migratory responses highly sensitive to properties of the chemotactic stimulus field.

2. Materials and methods

2.1. Cell culture

The rMC-1 cell line (Kerafast, Cat. No. ENW001) was maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L, 1 mM sodium pyruvate and 1500 mg/L sodium bicarbonate at 37C and supplemented with 10% fetal bovine serum (FBS) (Invitrogen-Gibco, Rockville, MD) in a 5% CO2 incubator. Cells were passaged with Accutase® Solution (Sigma Life Science). For experiments, cells were initially seeded at 10^6 cells/mL per T-25 flask in medium containing 10% FBS, then cultured in serum-restricted medium containing 1% FBS for 2 weeks as per phenotypic guidelines (Sarthy et al., 1998).

2.2. Cell morphology

Cell morphology was evaluated using Cell Shape Index (CSI), a dimensionless parameter that serves as a quantitative measure of asymmetry (Versaevel et al., 2012), shown in Equation (1):

$$CSI = \frac{4\,\Pi \boldsymbol{A_S}}{\boldsymbol{P}^2} \quad (1)$$

where A_s and P represent cell surface area and perimeter, respectively. Values of CSI range from 1.0 for an idealized circular shape to near 0 for cells that are narrow and linearly elongated.

2.3. Immunocytochemistry

Cells were plated at 2×10^5 cells/mL on borosilicate glass well plates (Lab-Tek, Cat. No.155383) in DMEM with 1%FBS and allowed to adhere overnight, then rinsed with PBS and fixed with 10% formalin (Sigma-Aldrich, Cat. No. HT501128) for 10 min. After rinsing twice with PBS, the samples were permeabilized with 0.1% Triton-X-100 in PBS (Sigma-Aldrich, Cat. No. X100) for 10 min, blocked for 60 min with 1% BSA, rinsed twice with the same blocking solution and exposed to 1:500 dilution of anti-GFAP (Abcam, Cat. No.7260) and 1:5000 dilution solution of anti-Nestin (Millipore, Cat. No., ABD69) consecutively overnight at 4 °C. The samples were then washed three times with 0.2% BSA in PBS and incubated with Alexa Fluor 594 anti-rabbit IgG (ThermoFisher, Cat. No., A11012) to detect Nestin and goat anti-rabbit IgG (H + L) Alexa Fluor 488 (ThermoFisher, Cat. No., A11008) for anti-GFAP samples. All samples were exposed to the secondary antibody at a concentration of 5 μg/mL for 30 min at 22 °C, and then rinsed twice with blocking solution. Nuclear staining (Life Technologies, Cat. No. R37605) was performed for 20 min at 22 °C, after which the samples were rinsed twice with PBS and covered in glycerol (Life Technologies, Cat. No.15514-011) for preservation.

2.4. Transwell migration assays

Transwell assays were used to measure the number of cells that migrated towards different concentrations of growth factors, as described previously by our group (Rico-Varela et al., 2015). The assay consisted of two compartments filled with medium and separated by an 8 micron-porous membrane (VWR, Cat. No.62406-198). Cells were seeded in the upper compartment and were allowed to migrate through the porous membrane into the lower compartment for 18 h at 37 °C, in 5% CO2 incubator. Approximately 1×10^6 cells/mL were seeded in 300 μl of DMEM complete medium (DMEM with FBS) in each upper chamber, while 700 μl of serum-free medium (DMEM only) was pipetted into each lower chamber. In addition, lower chambers had concentrations of the following growth factors: EGF (Sigma Aldrich, Cat. No. SRP3196) at 10, 100, and 500 ng/mL; FGF-8 (ThermoFisher, Cat. No. PHG0184) at 10, 100 and 1000 ng/mL; FGF-2 (Sigma Aldrich, Cat. No. SRP4039) at 10, 100 and 1000 ng/mL; and finally VEGF (Sigma Aldrich, Cat. No. SRP3182) at 10 and 100 ng/mL, which were diluted in DMEM. After incubation, cell numbers on the underside of the filter were estimated by staining using the Cyquant kit®, which yields absorbance values proportional to DNA content.

2.5. Reverse transcription quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) was used to measure expression levels of four receptor molecules and two glial cell markers: FGFR-2 (receptor for bFGF), FGFR-3 (receptor for FGF8), EGF-R (receptor for EGF), NRP-1 (the receptor for VEGF-A), as well as GFAP and Nestin (interfibrillar proteins of neural progenitors and/or glia (Bouameur and Magin, 2017)). RNA was isolated from 3 separate thawed stocks of rMC-1 cells using Trizol (Sigma-Aldrich) and measured photometrically. First