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Collective adhesion and displacement of retinal progenitor cells upon extracellular matrix substrates of transplantable biomaterials

Ankush Thakur1, Shawn Mishra1, Juan Pena1, Jing Zhou2,3, Stephen Redenti2,3,4, Robert Majeska1 and Maribel Vazquez1,4

Abstract
Strategies to replace retinal photoreceptors lost to damage or disease rely upon the migration of replacement cells transplanted into sub-retinal spaces. A significant obstacle to the advancement of cell transplantation for retinal repair is the limited migration of transplanted cells into host retina. In this work, we examine the adhesion and displacement responses of retinal progenitor cells on extracellular matrix substrates found in retina as well as widely used in the design and preparation of transplantable scaffolds. The data illustrate that retinal progenitor cells exhibit unique adhesive and displacement dynamics in response to poly-L-lysine, fibronectin, laminin, hyaluronic acid, and Matrigel. These findings suggest that transplantable biomaterials can be designed to improve cell integration by incorporating extracellular matrix substrates that affect the migratory behaviors of replacement cells.

Keywords
Neuroclusters, migration, microfluidics, photoreceptors, transplantation

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Introduction
Specialized biomaterials1–3 have been used in conjunction with extracellular matrix (ECM) proteins to introduce a variety of replacement cell types4–7 into damaged and/or injured retina8,9 via transplantation therapy. The microenvironments within such transplantable biomaterials provide structural and chemical cues that influence nearly all aspects of the cells that populate them, including de/differentiation,10 migration,11 intercellular connectivity,12 and long-term survival.13 Reported advancements14 in the survival and integration of transplanted photoreceptor progenitors have inspired dramatic innovation in contemporary transplantation strategies, including elastomeric grafts15 and protein-coated, electrospun fibers,16 whose properties mimic in vivo cues affecting cell fate, behavior, and response in host retina.17,18

A significant, but largely understudied, group of physical cues guiding biomaterial design for retinal transplantation are those which support and/or influence the migratory behavior of transplantable cells. This is surprising because the migration of transplanted cells is fundamental to cellular and synaptic integration within host retina. As illustrated in Figure 1, cells transplanted into the sub-retinal space must exit the biomaterial and navigate the complex retinal architecture via the outer nuclear layer in order to connect with neuronal targets of the host.8,19 Few studies have explored how properties of biomaterials affect outward cell migration into host retinal laminae. Such
migration processes are complex because they include movement of individual cells as well as the collective migration of neuroclusters. Collective behaviors of transplanted cells are particularly important because donor cells are explicitly derived to mimic earlier developmental states, where cells largely interact as neuroclusters. The mechanisms of collective cell adhesion and migration are also acutely affected by factors such as population density and interstitial interactions, both of which are routinely altered in order to increase the integration of transplanted cells.

Our group has previously evaluated the collective and individual migration of retinal progenitor cells (RPCs) and photoreceptor precursor cells (PPCs) toward ligand gradients derived from light-damaged retina. In this article, we quantify the clustering, adhesion, and displacement of RPCs in response to four ECM substrates present in developing and mature retina, and/or used extensively in the development and testing of transplantable biomaterials as described in Table 1: fibronectin (FN), laminin (LM), hyaluronic acid (HA), and Matrigel (MG). RPC behavior upon these substrates was also compared against poly-1-lysine (PLL). This study also incorporated controlled microfluidic systems to evaluate behaviors of individual and clustered RPCs at the microscale, comparable to the sub-retinal spaces that replacement cells encounter during transplantation. Results illustrate that ECM substrates affected the size of individually adhered cells, the ratio of collectively to individually adhered cells and the displacements of both. These data suggest that ECM substrates used in cell replacement strategies affect cell migration in specific ways that should be considered in the development of enriched biomaterials for retinal transplantation.

**Methods and materials**

**Cell isolation and culture**

Mouse RPCs were isolated from dissociated retinas of post-natal (P4) C57BL/6J mice, homozygous for expression of enhanced green fluorescent protein under control of the beta actin promoter (EGFP+/+) (gift from Dr Young, Harvard University). RPCs were expanded and maintained at 37°C in a 5% CO₂ incubator in neurobasal complete medium (Invitrogen-Gibco, Rockville, MD) containing 2 mM L-glutamine, 100 mg/mL penicillin-streptomycin, B27 and N2 neural supplements, and 20 ng/mL epidermal growth factor (Promega, Madison, WI) as described previously by our group. For testing, cells were detached with trypsin-EDTA, re-suspected in culture medium, counted, and seeded as described below.

**ECM substrates**

Mouse laminin (LM, Sigma-Aldrich, St. Louis, MO) and human fibronectin (FN, Thermo fisher, Waltham, MA) were used in this study.
MA) were dissolved and diluted to 10 μg/mL and 100 μg/mL, respectively, in phosphate-buffered saline (PBS). Growth factor-reduced MG (BD Biosciences, Franklin Lakes, NJ) was diluted to 100 μg/mL in PBS. HA (Alfa Aesar, Ward Hill, MA) was added to PBS at 30 mg/mL with thorough sonication and allowed to dissolve for 24 h at room temperature. PLL was used as a control substrate. Wells were coated with a 15 ng/mL solution in PBS at 5°C overnight and left to air dry in a laminar flow hood for 1 h prior to study. Surfaces of 12-well culture plates (VWR International, Bridgeport, NJ) were functionalized with a 1 mL volume of LM, FN, or MG per well and 0.5 mL of HA for 1 h. All wells were washed afterward with 1 mL of PBS. Substrates were functionalized at room temperature (25°C) for all solutions except LM, which was performed on ice as per vendor protocols. All wells were used on the day of surface functionalization.

**Morphology and adhesion density of individual RPCs**

RPCs were plated at (1) low cell density of 10⁴ cells/well; (2) moderate, but sub-confluent, density of 10⁵ cells/well; and (3) high cell density of 10⁶ cells/well that approached confluence in 12 well plates (~1 mL/well). The size and morphology of individually adhered cells were evaluated using the cell shape index, CSI, defined in equation (1) as a dimensionless parameter that quantitatively represents cell asymmetry:

$$CSI = \frac{4\pi A_S}{P^2}$$  \hspace{1cm} (1)

where $A_S$ represents the surface area of individually adhered cells and $P$ represents the cell perimeter. Values of CSI range from 1.0 for an idealized circular shape to 0.0 for cells that exhibit a perfectly linear elongation, as shown in the schematic of Figure 2. In this study, individual cells (i.e., not part of a neurocluster) were defined as those whose contact with neighboring cells was limited to either (1) extended, continuous interfacial contact with a single cell along the plasma membrane (e.g., daughter cells following mitosis) or (2) discrete point contacts via processes or extensions with one or more other cells. In addition, the average cell density of individually adhered cells was quantitatively represented by the cell adhesion density, $\pi_{ADH}$, defined in equation (2):

$$\pi_{ADH} = \frac{\sum_{i=1}^{N} A_{SC}}{N A_{STL}}$$  \hspace{1cm} (2)

Table 1. Extracellular matrix (ECM) compounds selected: a summary of significant ECM properties and their recent applications in vitro and in vivo.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Biochemistry</th>
<th>In vivo significance</th>
<th>In vitro systems</th>
<th>Biomaterials and transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin (FN)</td>
<td>230–270 kDa glycoprotein</td>
<td>Component of vascular basement membrane and retinal pigment epithelium (RPE)</td>
<td>Enhanced cell adhesion of cultured retinal cells</td>
<td>Injectable cell delivery for retinal transplantation</td>
</tr>
<tr>
<td>Laminin (LM)</td>
<td>400–900 kDa glycoprotein</td>
<td>Major component of retinal laminae</td>
<td>Testing of scaffold polymer-blended materials for photoreceptors</td>
<td>Transplanted retinal sheets of stem cells</td>
</tr>
<tr>
<td>Hyaluronic acid (HA)</td>
<td>10⁴ Da Linear polysaccharide</td>
<td>Principal component of interphotoreceptor matrix (IPM)</td>
<td>Component of biomimetic hydrogels</td>
<td>Transplantable material from de-cellularized retina</td>
</tr>
<tr>
<td>Matrigel (MG)</td>
<td>Trade name for mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma</td>
<td>Evaluated angiogenic compounds</td>
<td>Tested photoreceptor morphology prior to transplantation</td>
<td>Stem cell culture to achieve desired morphology prior to retinal transplantation</td>
</tr>
<tr>
<td>Poly-L-lysine (PLL)</td>
<td>Synthetic polymer that enhances electrostatic interactions</td>
<td>Used for nanoparticle delivery in retina</td>
<td>Constituent of tunable retinal hydrogels</td>
<td>Coating for implants used in rat optic tract</td>
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</tbody>
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</table>
where $A_{SC}$ denotes the area of individually adhered cells within a substrate region of interest, $i$, and $A_{SA}$, $i$ denotes the surface area of that region of interest.

**Mean size and adhesion ratio of retinal neuroclusters**

Retinal neuroclusters were defined as groups of three or more cells with continuous and extended interfacial contact along their plasma membranes, as described per Figure 2. The mean size of each neurocluster, $X_{CL}$, was measured using its projected substrate surface area. The ratio of neuroclusters to individually adhered cells was assessed via a parameter called the adhesion ratio, $R_{ADH}$, defined in equation (3)

$$R_{ADH} = \frac{A_{NC}}{A_{SC}, A_{NC}} \times 100\%$$

where $A_{NC}$ is the projected surface area of adhered neuroclusters within a substrate region and $A_{SC}$ represents the total surface area of singly adhered cells. In this way, $R_{ADH}$ denotes the percentage of total cell-adhered surfaces that contain neuroclusters.

**Expression of adhesion receptors**

Expression levels of four genes encoding adhesion receptors were measured using quantitative polymerase chain reaction (qPCR) for integrin α3, integrin α7, integrin β3, and the adhesion molecule CD44 with primers shown in Table 2. Primer specificity was verified using Basic Local Alignment Search Tool (BLAST), which confirmed the selected forward and reverse primers listed. RNA was isolated from cells using Trizol (Sigma-Aldrich, St. Louis, MO) and measured photometrically. First-strand complementary DNA (cDNA) synthesis was performed using random hexamers followed by amplification with specific primers on a Rotor Gene 6000 thermal cycler (Qiagen, Inc., Germantown, MD) as per manufacturer instructions. The following amplification conditions were used: 95°C denaturation for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a hold at 4°C. Raw data were analyzed with Software version 2.2.3 (Qiagen Inc.) to determine the cycle threshold (CT) setting for assigning baseline and threshold CT determination. Relative expression (RE) of the sample gene was calculated using the conventional ΔΔCT method.

**Adhesion and displacement within microfluidic environments**

A previously described microfluidic system from our group, the μLane, was used in this study to evaluate RPC adhesion and displacement upon different ECM protein substrates. As shown in Figure 3, μLane systems comprised channels of 150-μm diameter and 12-mm length, as fabricated via conventional lithography, PDMS (poly(di)methyl-siloxane) micromolding, and glass bonding. The μLane inner surfaces were functionalized with LM, FN, HA, or MG at the concentrations described earlier. Solutions of RPCs at three different seeding densities of $10^4$, $10^5$, or $10^6$ cells/mL were then inserted into the μLane interstitial spaces. Cells within the μLane were imaged every 24 h for 3 days using phase-contrast microscopy.
Cell imaging and analysis

Phase-contrast images of adhered RPCs and neuroclusters were evaluated using a 20× objective lens (Nikon TE2500 microscope, Morrell Instruments, NY). Images from well plates were gathered from five non-overlapping fields via a conventional checkerboard pattern,55 while images from μLane systems were captured from five channel and reservoir areas. Size and morphology of individual RPC and cell aggregates were quantitated using ImageJ shareware (NIH, Bethesda, MD) as described previously by our group.24

Table 2. Gene regulation examined via quantitative polymerase chain reaction (qPCR): a listing of the genes encoding cell and surface adhesion molecules studied, alongside primer sequence, size in base pairs (bp), and accession number.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’−3’)</th>
<th>bp</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin α3 (ITGA3)</td>
<td>F: ACTACAGGC Guang ACATCACC R: AAGAAGCC Guang AGACAGA</td>
<td>20</td>
<td>NM_001306071.1</td>
</tr>
<tr>
<td>Integrin α7 (ITGA7)</td>
<td>F: TTGTGTAGCCAGATCAG R: TGATGTAGGAGAACGGAAGG</td>
<td>20</td>
<td>NM_008398.2</td>
</tr>
<tr>
<td>Integrin β3 (ITGB3)</td>
<td>F: TGAACACAGGAGGTTGGAAG R: GATGACGAACGCAATGAGC</td>
<td>20</td>
<td>NM_016780.2</td>
</tr>
<tr>
<td>CD44 (CD44)</td>
<td>F: TGAATCAAGGAGGTGAAGG R: AGCTTTCCTCTCGACCACA</td>
<td>20</td>
<td>NM_001177787.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AACATTTGGCATTTGGAAAGG R: ACACATTTGGGGGTAGGAACA</td>
<td>20</td>
<td>NM_001289726.1</td>
</tr>
</tbody>
</table>

Figure 3. The μLane system. (a) Projection images of μLane construction, comprising two layers of PDMS polymer ((1), (2): poly-dimethyl siloxane) ozone-bonded upon a (3) glass coverslip. The μLane systems has a channel length of l = 1.2 cm, reservoir volume of V = 0.5 mL, and PDMS thickness of t = 0.75 cm. (b) Image of closed μLane system with highlighted microchannel. Scale = 1 mm.

Statistical analysis

Differences among adherent cell groups were evaluated using one-way analysis of variance (ANOVA) and post hoc test (Tukey). A one-way ANOVA test at the 95% confidence interval assessed statistical significance across biomaterial substrates and cell seeding densities, while the post hoc test (Tukey) determined the disparity among different groups. Calculated p-values < 0.05 were considered statistically significant and denoted by an asterisk. The numbers of experiments performed for each test were at least 10 per condition.

Results

Adhesion of RPCs

Adhered RPCs were evaluated 2 days post-seeding upon substrate surfaces of PLL, FN, LM, HA, and MG, as shown in Figure 4. RPCs adhered to all substrates in different combinations of single cells and neuroclusters (defined in Figure 2), each with varying morphology and mean size. No significant differences in viable cell numbers were observed among RPCs adhered to substrates, individually or within neuroclusters, as measured via MTT assays and conventional DAPI staining (data not shown).

Retinal progenitor morphology (CSI) and adhesion density (ΠADH)

Cells that did not adhere as part of neuroclusters exhibited varying cell morphology and density with biomaterial substrate. Changes in cell morphology were measured via the CSI shown in Figure 5(a). As seen, only morphology changes measured upon LM were statistically significant (p < 0.05), as RPCs became increasingly elongated with higher seeding density. RPCs upon LM demonstrated the only statistically significant elongation, as average values of CSI upon LM decreased dramatically from CSI = 0.67 at low seeding density (10^4 cells/mL) to CSI = 0.33 for high seeding density (10^6 cells/mL). There was no statistical
Figure 4. Aggregation and adhesion of retinal progenitor cells (RPCs). Images of RPCs at a cell density of $10^5$ cells/mL adhered as individual cells and neuroclusters upon surfaces of: (a) poly-L-lysine (PLL), (b) fibronectin (FN), (c) laminin (LM), (d) hyaluronic acid (HA), and (e) Matrigel (MG). Representative cell clusters are outlined per image and arrows point to individually adhered cells. Scale bar = 200 µm. Representative images of RPCs evaluated as (f, g) individually adhered cells, (h) RPCs adhered with close adhesion spacing but not as neuroclusters, and (i) RPCs adhered as part of neuroclusters. Scale bar = 20 µm.

Figure 5. Metrics of individual cell adhesion. The (a) average cell shape index (CSI) of retinal progenitor cells (RPCs) adhered as individual cells upon poly-L-lysine (PLL), fibronectin (FN), laminin (LM), hyaluronic acid (HA), and Matrigel (MG) at cell densities of $10^4$, $10^5$, and $10^6$ cells/mL. (b) Average values of single cell adhesion density, $\Pi_{ADH}$, upon the same biomaterial substrates. Statistical significance within bracketed values is denoted by an asterisk. The "#" symbol indicates a cell monolayer on FN.
Upon HA. Furthermore, values of $\text{RADH}$, and MG, but were highest at moderate seeding density measured by the adhesion ratio, $\text{RADH}$, both biomaterial substrate and cell seeding density, as clusters (vs individually adhered cells) also varied with seeding densities studied. Different between each biomaterial substrate across all $p < 0.05$) on PLL, FN, LM, and a complete RPC monolayer was observed upon FN. Mean values of calculated parameters are summarized in Table 3.

### Expression of adhesion receptors

The observed changes in the adhesive behavior of RPCs were evaluated in relation to expression of a panel of cell surface adhesion molecules known to serve as receptors for one or more of the ECM materials used as substrates in this study: integrin $\alpha_3$, integrin $\alpha_7$, integrin $\beta_3$, and CD44. As noted in Table 4, integrin $\alpha_3^{60-65}$ and integrin $\alpha_7^{66-69}$ bind to FN and LM, but CD44 is the receptor for HA, and integrin $\beta_3^{70-74}$ is expressed by cells of the retinal pigment epithelium (RPE) and highly implicated in retinal angiogenesis. We observed that RPCs seeded onto substrates identified as ligands expressed somewhat higher levels of most receptors than when seeded onto PLL. Figure 7 shows that integrin $\beta_3$ was up-regulated by FN and LM, but down-regulated by HA and MG, and CD44 was down-regulated by FN and LM, but up-regulated by HA and MG as per the $\Delta\Delta\text{CT}$ method.

### Attachment and displacement of RPCs

To evaluate RPC adhesion and displacement within the microscale of sub-retinal spaces, cell suspensions were seeded into $\mu$Lane devices previously functionalized with FN, LM, HA, or MG. RPCs were seen to adhere as neuroclusters, individual cells, or combinations of the two, depending on the substrate used. As shown in Figure 8, RPCs did not aggregate into neuroclusters within $\mu$Lane devices functionalized with FN or MG, but rather adhered solely as individual cells. By contrast, RPCs were observed

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**Table 3. Summary of parameters measured for adhered retinal progenitor cells (RPCs).**

<table>
<thead>
<tr>
<th>Matrix used</th>
<th>CSI (mean)</th>
<th>$\langle\Pi_{\text{ADH}}\rangle$ (mm$^{-2}$) (mean)</th>
<th>$\bar{X}_{\text{CL}}$ (mm) (mean)</th>
<th>$R_{\text{ADH}}$ (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-lysine (PLL)</td>
<td>0.46 ± 0.09</td>
<td>20.8 ± 1.8</td>
<td>5.1 ± 0.55</td>
<td>56.3 ± 3.5</td>
</tr>
<tr>
<td>Fibronectin (FN)</td>
<td>0.36 ± 0.02</td>
<td>22.5 ± 2.4</td>
<td>1.8 ± 0.38</td>
<td>22.8 ± 3.9</td>
</tr>
<tr>
<td>Laminin (LM)</td>
<td>0.50 ± 0.14</td>
<td>10.2 ± 0.34</td>
<td>4.6 ± 0.75</td>
<td>59.1 ± 4.8</td>
</tr>
<tr>
<td>Hyaluronic acid (HA)</td>
<td>0.48 ± 0.04</td>
<td>10.7 ± 0.84</td>
<td>7.6 ± 0.94</td>
<td>72.8 ± 4.0</td>
</tr>
<tr>
<td>Matrigel (MG)</td>
<td>0.38 ± 0.04</td>
<td>14.37 ± 0.64</td>
<td>10.1 ± 0.60</td>
<td>72.6 ± 4.74</td>
</tr>
</tbody>
</table>

Average values of individual cell shape index (CSI) and single cell adhesion density ($\Pi_{\text{ADH}}$) summarized alongside values of mean cluster size, $\bar{X}_{\text{CL}}$, and adhesion ratio, $R_{\text{ADH}}$, for RPCs adhered upon PLL, FN, LM, HA, and MG.

---

significance in CSI values among and across the other biomaterial groups ($p > 0.5$).

In contrast, the density of cells adhered as individuals rather than in clusters, $\Pi_{\text{ADH}}$, varied depending on both the biomaterial substrate and the initial number of cells plated. Changes in $\Pi_{\text{ADH}}$ were statistically significant across cell seeding density for LM, HA, and MG, as per Figure 5(b). Average $\Pi_{\text{ADH}}$ values were greatest upon LM and HA at moderate seeding density (10$^5$/mL), but steadily increased upon MG. By contrast, increases in $\Pi_{\text{ADH}}$ were most pronounced upon PLL and FN, where at high seeding density (10$^6$/mL) the largest $\Pi_{\text{ADH}}$ value was measured upon PLL, and a complete RPC monolayer was observed upon FN. Mean values of calculated parameters are summarized in Table 3.

**Size ($\bar{X}_{\text{CL}}$) and extent ($R_{\text{ADH}}$) of RPC neuroclusters**

Neuroclusters in Figure 4 exhibited a range of mean sizes, $\bar{X}_{\text{CL}}$, as measured by projected surface area. Qualitatively, RPC neuroclusters appeared compact and circular upon PLL and LM, but more diffuse and irregularly shaped neuroclusters upon FN, HA, and MG. The data in Figure 6(a) illustrate that the highest mean cluster sizes were measured upon MG and HA at high (10$^6$/mL) and moderate (10$^5$/mL) seeding densities, respectively (excluding the FN monolayer). Mean sizes of retinal neuroclusters, $\bar{X}_{\text{CL}}$, rose with increasing cell density ($p < 0.05$) on PLL, FN, LM, and MG, but were highest at moderate seeding density upon HA. Furthermore, values of $\bar{X}_{\text{CL}}$ were statistically different between each biomaterial substrate across all seeding densities studied.

The percentage of cell-occupied area containing neuroclusters (vs individually adhered cells) also varied with both biomaterial substrate and cell seeding density, as measured by the adhesion ratio, $R_{\text{ADH}}$, in Figure 6(b). Values of average $R_{\text{ADH}}$ increased with cell seeding density upon FN, HA, and MG and decreased with seeding density upon PLL and LM. The highest values of $R_{\text{ADH}}$ were measured upon both HA and MG at the highest seeding densities (10$^6$/mL), where 85% of adhered surface areas contained neuroclusters. As previously noted, RPCs formed a complete monolayer on FN at high seeding density rather than discrete neuroclusters. Conversely, the lowest adhesion ratio of $R_{\text{ADH}}$=31% was measured upon FN at low cell seeding density (10$^4$/mL), where less than one third of cells adhered as part of neuroclusters. Furthermore, RPCs within adherent neuroclusters exhibited similar morphologies upon all biomaterials, with an average CSI = 0.82±0.4 that was significantly higher (indicative of more rounded cells) than that measured for any individually adhered cell group (Figure 5(a)). Mean values of calculated parameters are summarized in Table 3.

The observed changes in the adhesive behavior of RPCs were evaluated in relation to expression of a panel of cell surface adhesion molecules known to serve as receptors for one or more of the ECM materials used as substrates in this study: integrin $\alpha_3$, integrin $\alpha_7$, integrin $\beta_3$, and CD44. As noted in Table 4, integrin $\alpha_3^{60-65}$ and integrin $\alpha_7^{66-69}$ bind to FN and LM, but CD44 is the receptor for HA, and integrin $\beta_3^{70-74}$ is expressed by cells of the retinal pigment epithelium (RPE) and highly implicated in retinal angiogenesis. We observed that RPCs seeded onto substrates identified as ligands expressed somewhat higher levels of most receptors than when seeded onto PLL. Figure 7 shows that integrin $\beta_3$ was up-regulated by FN and LM, but down-regulated by HA and MG, and CD44 was down-regulated by FN and LM, and up-regulated by HA and MG as per the $\Delta\Delta\text{CT}$ method.
Table 4. Summary of adhesion molecules examined alongside their applications to neural cells in the retina.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>In vivo significance</th>
<th>Biomaterials and testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin</td>
<td>α3</td>
<td>Integrins in neuro human retina\textsuperscript{40,61}</td>
</tr>
<tr>
<td></td>
<td>α7</td>
<td>Critical for retinal synapses\textsuperscript{52}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adhesion receptor for LM needed in synaptic transmission\textsuperscript{43}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mediates neurite outgrowth\textsuperscript{57}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implicated in development\textsuperscript{68}</td>
</tr>
<tr>
<td>Integrin</td>
<td>β3</td>
<td>Inhibition disrupts development\textsuperscript{70}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed by RPE\textsuperscript{71}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implicated in retinal angiogenesis\textsuperscript{72,73}</td>
</tr>
<tr>
<td>CD 44</td>
<td></td>
<td>Adhesion molecule within interphotoreceptor matrix\textsuperscript{75}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Critical for retinal development\textsuperscript{16,77}</td>
</tr>
</tbody>
</table>


to adhere within μLane systems functionalized with LM and HA as both single cells and neuroclusters. As summarized in Table 5, neuroclusters within μLane systems functionalized with LM and HA exhibited net displacements of 18–26 μm (i.e., two diameters of individually adhered cells), while that of individually adhered RPC within LM- and HA-treated
μLane substrates was 3–6 μm, or less than one cell diameter. Individual RPCs adhered upon FN- and MG-treated devices underwent net displacements of 4–9 μm.

**Discussion**

This study examined the ability of biomaterial substrates used in transplantable biomaterials to alter RPC adhesion, clustering, and displacement. The balance between cell adhesion and motility is a critical consideration in the design of retinal scaffolds, as insufficient adhesion can result in cell death while overly strong adhesion may adversely affect RPC morphology and differentiation or prevent cell migration needed to re-establish functional connectivity. Moreover, since RPCs migrate as both clusters and individual cells during retinogenesis, it is also important to know the extent to which retinal scaffolds can mimic this behavior.

This study defined two parameters, adhesion ratio, $R_{adh}$, and adhesion density, $\Pi_{adh}$, which quantitatively represented the ratio of individually adhered cells to adhered neuroclusters and the portion of total substrate area covered by cells, respectively. Results illustrate that both parameters differed depending on both ECM substrate and seeding density used. Of note, increasing the number of RPCs seeded on a substrate generally resulted in increased numbers of single cells (Figure 5) but did not necessarily yield proportionately larger neuroclusters. Rather, larger seeding densities produced (1) larger numbers of moderately sized neuroclusters upon HA and LM, (2) elicited no change in neurocluster size or adhesion upon PLL, and (3) produced larger numbers of larger-sized neuroclusters upon MG (Figure 6). Interestingly, on most substrates, formation of neuroclusters, even at the highest density examined, resulted in substantial areas of open space not covered by either clusters or single cells. This suggests that the interactions between cells and substrate were notably weaker than those between cells. In contrast, cells plated on FN at high density formed a complete monolayer, indicating that cell–substrate interaction was predominant. The implications of these differences for RPC adhesion and migration, either as single cells or clusters, have yet to be established, although the ability to characterize clustering behavior, quantitatively, will facilitate this process.

Expression of adhesion receptors as assessed by polymerase chain reaction (PCR) was altered when RPCs were cultured on their respective ligands (Figure 7). However, the substrate-dependent differences in adhesion receptor expression were modest. Consequently, while the receptors examined are likely to contribute to changes in cell shape, formation of neuroclusters, or the displacement of either individual cells or clusters, we could not establish a clear relationship between those processes and receptor expression levels. That expression of adhesion receptors, such as integrins, can be modulated in response to the amount of available ligand (substrate) in ECM has been

![Figure 7. Differences in gene expression of adhered RPCs. Fold change in regulation of the genes ITGA3, ITGA7, ITGB3, and CD44 that encode (a) integrin α3, integrin α7, integrin β3, and CD44 upon adhesion with fibronectin (FN), laminin (LM), hyaluronic acid (HA), and Matrigel (MG). (b) Schematic representation of up- and down-regulation of encoding genes as measured via the ΔΔCT method.](image-url)
well established. However, differences in gene expression may underestimate changes in the number and organization of adhesion receptors on cell surfaces. In addition, averaging techniques like PCR may not be sensitive enough to detect subtle expression changes in mixed populations such as RPCs present both as clustered and individually adhered cells. Furthermore, it has been reported that cell adhesion even to single-component extracellular matrices, such as LM or FN, may involve multiple integrin family members while CD44 is capable of binding both HA and collagen. Hence, adhesion to the various ECM substrates in this study may have activated more than one receptor, including some outside the limited group whose expression was examined. Future study will examine expression of molecules able to mediate cell–cell interactions that aid formation and stabilization of cell clusters, for example, cadherins.

The use of the μLane system to evaluate the microscale displacement of mixed (individual and clustered) RPCs (Figure 2) within microfluidic environments represents a novel application of technology little used in retinal cell replacement studies despite widely reported usefulness in a variety of cell-based systems (Figure 3). Our system readily enables quantitative study of cell migration over sub-retinal distances <50 μm as well as distances >1000 μm for chemotaxis driven by extrinsic fields, as shown previously by our group.11,26,27 Our studies demonstrated that RPCs formed neuroclusters and underwent displacement both as individual cells and as clusters within the interstitial spaces of the μLane functionalized with LM or HA, although FN- and MG-treated substrates exhibited single-cell adhesion, only (Figure 8). Why RPCs did not form neuroclusters on FN and MG in the μLane system, as they did in bulk culture, is so far unclear, but suggests differences in substrate composition or organization between the microfluidic and bulk microenvironments that warrant further investigation. In any case, the absence of clustering suggests a predominance of cell–matrix over cell–cell interactions (Table 3), as previously noted.

Table 5. Retinal progenitor adhesion and displacement within μLane system.

<table>
<thead>
<tr>
<th>Matrix used</th>
<th>Neurocluster formation</th>
<th>Single cell displacement (μm)</th>
<th>Neurocluster displacement (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin (FN)</td>
<td>No</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>Laminin (LM)</td>
<td>Yes</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Hyaluronic acid (HA)</td>
<td>Yes</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>Matrigel (MG)</td>
<td>No</td>
<td>9</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 8. Displacement of adhered retinal progenitor cells (RPCs) within the μLane microfluidic system. Images describing attachment of RPC cells and/or neuroclusters within (a) μLane systems whose inner surfaces were functionalized with (b) fibronectin (FN), (c) laminin (LM), (d) hyaluronic acid (HA), and (e) Matrigel (MG). Scale bar = 75 μm.
Finally, the displacements observed by RPCs as both single cells and clusters on LM and HA were clearly measurable and distinct. We also note that those displacements were observed in the absence of applied stimuli, indicating that chemotactic or chemokinetic responses of RPCs to chemical and physical signals are readily amenable to study. Why the observed displacement of neuroclusters was greater than that of single cells is not known (Table 3). While quantitative study of collective migration is beyond the scope of this article, the size and composition of neuroclusters is known to affect migration modalities\textsuperscript{20,93,94} which may directly influence cell distances traveled. Furthermore, different ECM substrates may preferentially regulate cell adhesion molecules that maintain the cohesion of motile clusters\textsuperscript{93} and whose interactions may outweigh the cell–matrix adhesion studied here. In any event, our results suggest that, among the substrates examined, LM may be preferred for retinal cell transplantation. LM is present in mature and developing retina, RPCs express cell surface LM receptors, and LM mediates adhesion both as single cells and as neuroclusters. These features suggest that LM matrices may enable tuning to achieve both collective and single cell migration with desirable displacement distances. Future study will benefit from using known physical and chemical cues of retinal injury and/or neurodegeneration to directly evaluate RPC migratory behavior into injured and/or damaged laminae.

**Conclusion**

Composites of ECM substrates and biomaterials have evolved to support increasing numbers of cells, extend cell survival, and promote cell adhesion as strategies to improve outcomes of transplantation therapy. This study evaluated the abilities of selected substrates to regulate RPC adhesion, shape, and cell displacement as individual cells and as aggregates to design and/or enhance transplantable biomaterials. Results illustrate that combinations of clustered and individual cells on different substrates led to different behaviors that may influence the ability of transplanted, or replacement, cells to migrate effectively and achieve connectivity with host cells in retinal laminae. These findings suggest that by characterizing and/or manipulating the migratory behaviors of replacement cells, transplantation strategies can be enriched to improve cell integration.

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