

Myelin-associated proteins block the migration of olfactory ensheathing cells: an *in vitro* study using single cell tracking and traction force microscopy.

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Summary

Newly generated olfactory receptor axons grow from the peripheral to the central nervous system aided by olfactory ensheathing cells (OECs). Thus, OEC transplantation has emerged as a promising therapy for spinal cord injuries and for other neural diseases. However, these cells do not present a uniform population, but, instead, a functionally heterogeneous population that exhibits a variety of responses including adhesion, repulsion and crossover during cell-cell and cell-matrix interactions. Some studies report that the migratory properties of OECs are compromised by inhibitory molecules and potentiated by chemical gradients. Here, we demonstrated that rodent OECs express all the components of the Nogo Receptor complex and that their migration is blocked by Myelin. Next, we used cell tracking and traction force microscopy to analyze OEC migration and its mechanical properties over Myelin. Our data relate the absence of traction force of OEC with lower migratory capacity, which correlates with changes in the F-Actin cytoskeleton and focal adhesion distribution. Lastly, OEC traction force and migratory capacity is enhanced after cell incubation with the Nogo Receptor inhibitor NEP1-40.

Introduction

The olfactory system in the adult nervous system is renewed throughout life with the help of olfactory ensheathing cells (OECs). The elongation of newly generated olfactory receptor axons between the peripheral and central nervous system may be largely attributed to the properties of OECs that ensheath and guide the axons of olfactory neurons that extend from the olfactory epithelium to the olfactory bulb (Doucette, 1995; Ramon-Cueto and Valverde, 1995). Given their axon growth-promoting properties, natural or genetically modified OECs have been studied extensively and transplanted into the injured spinal cord to promote axonal regeneration (Ramon-Cueto and Nieto-Sampedro, 1994; Li et al., 1997; Ramon-Cueto et al., 1998; Navarro et al., 1999; Ramon-Cueto et al., 2000; Ruitenbergh et al., 2002; Garcia-Alias et al., 2004; Moreno-Flores et al., 2006). Thus, OEC transplantation has emerged as a promising therapy for spinal cord injuries and for other neural diseases (Moreno-Flores and Avila, 2006; Raisman, 2007; Ramon-Cueto and Munoz-Quiles, 2010). Several OEC subpopulations have been identified in the olfactory system on the basis of their topographical distribution, distinctive morphology, intracellular cytoskeletal distribution and antigenic or gene expression profiles (Au and Roskams, 2003; Vincent et al., 2005; Guerout et al., 2010; Windus et al., 2010). Indeed, two populations of OECs have been described *in vitro* and in some *in vivo* studies: Schwann cell-like OECs (sOECs), with fusiform bipolar form; and astrocyte-like OECs (aOECs), with a more flattened structure (Franceschini and Barnett, 1996; Kumar et al., 2005). In fact, the morphology of cultured OECs is dependent on the culture media (Au and Roskams, 2003; Higginson and Barnett, 2011); although they transform from a fusiform to a flat-sheet form spontaneously (Huang et al., 2008).

It has been suggested that sOECs have higher regenerative potential than aOECs (Ramon-Cueto et al., 1993; Franklin et al., 1996; Imaizumi et al., 1998). This property has been associated with the greater migratory potential of the former. In addition, different migratory properties have also been reported after transplantation in lesioned central nervous system (CNS) (Gudino-Cabrera et al., 2000; Nieto-Sampedro, 2003). Hence, Lu et al. failed to find unique migratory properties of OECs when these cells were implanted into spinal cord lesion (Lu et al., 2006). These results suggest that migration of OECs is modulated by specific interactions with the substrate (Saxton, 2007). In the injured spinal cord, lesioned axons are confronted with a changing environment with huge variety of growth inhibitory molecules located in the meningo-

glial scar and adjacent spinal cord regions (Silver and Miller, 2004; Fawcett, 2006). OEC migration *in vitro* is modulated by neurotrophins, such as the glial cell-derived neurotrophic factor (GDNF), or chemicals such as lysophosphatidic acid (LPA) (Yan et al., 2003; Cao et al., 2006; Windus et al., 2011). In addition, other secreted molecules involved in cell and axon migration, such as Slit-2, negatively modulate OEC migration (Huang et al., 2011). However, although further studies are needed, it seems that Slit-2 is not over-expressed after spinal cord lesion (Wehrle et al., 2005), in contrast to Slit-1 and Slit-3 (Wehrle et al., 2005), semaphorins (Pasterkamp et al., 1999), Netrin-1 (Low et al., 2008), chondroitin sulphate proteoglycans (Bradbury et al., 2002) and Myelin-associated inhibitors (MAIs) (Schwab, 1990). However, OECs show changes in their intracellular signaling mechanisms (Cofilin phosphorylation, RhoK activity and changes in Ca^{2+} stores) after Slit-2 treatment (Huang et al., 2011) that are common to other inhibitory systems (Silver and Miller, 2004). In fact, these observations raised the notion that OECs respond to a wide range of molecules. Indeed, Nogo-A (a MAI) enhances the adhesion of OECs affecting their migration (Su et al., 2007).

Directed cell migration requires the spatial and temporal coordination of cell adhesion and protrusion with the generation of traction forces at the cell/extra-cellular matrix (ECM) interface (Lauffenburger and Horwitz, 1996). These traction forces are mainly generated by the acto-myosin cytoskeleton and transmitted to the ECM through focal adhesions (FAs) (Balaban et al., 2001; Beningo et al., 2001; Bershadsky et al., 2003). Thus, here we examined the effects of MAIs on rodent OEC migration by using single cell tracking imaging on micropatterned substrates and traction force microscopy (TFM). First, we found that OECs express all the molecular machinery required to transduce the inhibitory effects of MAIs and that the migratory properties of OECs were largely blocked by MAIs. Moreover, using TFM, we quantitatively demonstrate that OECs largely decrease their traction stress over Myelin, a finding that correlates with decreased focal contacts and redistribution of F-actin cytoskeleton. Finally, we show that the incubation of OECs with the Nogo Receptor 1 (NgR1) blocking peptide NEP1-40 partially overcomes MAI-mediated migratory inhibition, thereby restoring the traction forces of these cells and their cytoskeletal organization.

Results

TEG3 is a clonal OEC line that shows similar growth-promoting capacity to non-modified OECs (Moreno-Flores et al., 2003). In addition, this cell line shows all the types of morphology described for primary OECs in culture (Moreno-Flores et al., 2006; Pastrana et al., 2006). In a first set of experiments, we characterized the TEG3 cell line in our culture conditions (Fig. 1). In these conditions, fusiform sOEC forms were predominant over aOEC ones (Fig. 1A-D). Specific markers of OECs (p75, S100 β or GFAP) were present in our cultured cells (Fig. 1A-D). Next, using Western blotting, we examined whether TEG3 cells express members of the common receptor machinery for MAIs (Nogo receptor complex) (Yamashita et al., 2005; Zhang et al., 2008; Llorens et al., 2011). Indeed, the revealed blots demonstrated that p75, NgR1, TROY and Lingo1 were expressed by cultured TEG3 cells (Fig. 1E). This complex was functional since incubation with Myelin increased activated RhoA as well as ERK 1-2 phosphorylation (supplementary material Fig. S1). In a second set of experiments, we cultured these cells in 35 mm \varnothing culture dishes patterned with dots of Myelin tagged with 0.75 μm \varnothing FITC beads. After Phalloidin-Alexa594 incubation and DAPI counterstaining, cells were exclusively located in regions not containing Myelin-Alexa594 (Fig. 1F). To avoid unspecific effects produced by FITC-tagged microbeads on OEC adhesion, we performed a stripe assay using brain Myelin extract as substrate (Fig. 1F,H). Stained cells were observed homogenously in stripes containing Poly-L-Lysine/Poly-L-Lysine and BSA-Alexa594-Laminin (Fig. 1G). In contrast, cells did not adhere to stripes containing Myelin compared to Poly-L-Lysine (Fig. 1H). Next we performed a multiple cell migration analysis of TGE3 cells growing over Poly-L-Lysine + Laminin- or Poly-L-Lysine + Myelin-coated substrates (Fig. 2A,B). Low density TEG3 cultures were monitored for 20 hours (20x objective; 1 frame every 8 minutes) in an inverted Olympus IX-71 microscope equipped with a cooled fluorescence camera and a cell culture incubation chamber (see Materials and Methods for details). The migration speed of OECs grown on Myelin-coated substrates (0.01 $\mu\text{g}/\mu\text{l}$ and 0.02 $\mu\text{g}/\mu\text{l}$) showed a decrease of 48% and 56% respectively compared to those on Laminin coated substrate (Fig. 2C). In contrast, cell migratory capacity was restored (up to 92% of the Laminin migration value) by culture in heat-denatured Myelin-coated substrate (Fig. 2C). A plot of the x/y cell trajectories revealed higher persistence on Laminin-coated than on Myelin-coated substrate (Fig. 2D).

To systematically analyze cell migration, we computed the mean square displacement (MSD) of cell trajectories. We then fitted the MSD to a power law expression: $MSD = D \times \Delta t^\beta$, where D is a scaling parameter and β indicates the degree of persistence. When $\beta=1$ the motion is random, when $\beta<1$ the motion is subdiffusive (anti-persistent), and when $\beta>1$ the motion is superdiffusive (persistent) (Bursac et al., 2005). For each time interval studied, cells on Laminin-coated substrates migrated further than those on Myelin-coated (Fig. 2E). To assess the degree of persistence as a function of the time interval, we calculated the local value of β as: $\beta = \partial \log(MSD) / \partial \log(\Delta t)$ (Bursac et al., 2005). Cells on Myelin-coated substrates exhibited a slightly subdiffuse behavior regardless of the time interval studied. In contrast, those on Laminin-coated substrates moved in a slightly superdiffusive manner at short time intervals but their trajectories became progressively subdiffusive with increasing time (Fig. 2E). Taken together, these data indicate that while cells on Laminin-coated substrates were more motile than those on Myelin-coated, they oscillated around their initial position over long intervals (Fig. 2D).

High power observation of cells growing on Laminin and Myelin showed the specific details of their migration (Fig. 2F). Cells growing on Laminin-coated substrate migrated in a similar manner to a fibroblast-like cell with dynamic lamellipodia and further nuclear translocation to the cell leading edge, leaving a tail process (Fig. 2F, upper panels). In fact, the cells translocated the nuclei between rostral to caudal cell locations following the largest lamellipodia. However, cells growing on Myelin displayed decreased lamellipodia dynamics and motile lamellipodia were observed in several cell domains (Fig. 2F, lower panels). This finding contrasts with the behavior of cells growing on Laminin, where two main lamellipodia were located on the opposite sides of the fusiform cell, with few transient lamellipodia that displaced laterally along the cell (see also Fig. 3D,E).

Next, we analyzed the migration distance of identified cells growing on Myelin in the absence or presence of the NgR1 inhibitor peptide NEP1-40, to block MAIs effects (Fig. 2G,H). Non-treated cells migrated shorter distances, showing a decrease of 49% compared to those on Laminin coated substrate. These distances increased by 20% after addition of the peptide (Fig. 2H).

Cells generate traction forces against their substrate during adhesion and migration, and traction forces are used, in part, by cells to sense the substrate. Thus, using TFM, we aimed to determine the distribution of traction forces in OECs cultured on a well characterized polyacrylamide (PAA) gel, which is linearly elastic, optically clear and amenable to protein conjugation (Califano and Reinhart-King, 2010) (Fig. 3). First we cultured OECs in PAA gels containing Laminin (2 $\mu\text{g}/\text{ml}$) or Myelin (0.02 $\mu\text{g}/\mu\text{l}$) and performed time-lapse analysis as above for 10 hours (Fig. 3A,B). TEG3 cells growing on PAA coated with Laminin or Myelin displayed slightly higher migration speed to those cultured on coated glass culture plates (Fig. 3C). Cultured cells over PAA containing Laminin or Myelin showed similar morphologies than those cultured on glass (Fig. 3D,E). MSD analysis indicates that cells on Laminin-coated substrates (glass or PAA) showed similar motion properties being more motile than those on Myelin-coated (Fig. 3F,G).

Using TFM we observed that cells seeded on Laminin-coated substrates transferred higher strain energy (0.037 ± 0.0061 , PJ) to their underlying substrate than those seeded on Myelin-coated (0.0033 ± 0.0007 , PJ) (Fig. 4A,B). Treatment with NEP1-40 induced a significant increase (2.75 fold increase) in strain energy (0.0091 ± 0.0016 , PJ) (Fig. 4A,B). These findings suggest that increased traction force generation provides OECs with greater migratory capacity. To study the mechanisms underlying changes in traction force generation in Laminin vs Myelin substrates as well as the effect of Nogo receptor blockage by NEP1-40, we examined the intracellular distribution of F-Actin (Fig. 5A,B) and FAs (Vinculin) (Fig. 5C-F). Results indicate the negligible presence of Phalloidin-positive stress fibers and FAs in TEG3 cells cultured on Myelin substrates in contrast to Laminin (Fig. 5). In addition, the distribution of FAs in cells incubated with NEP1-40 was partially recovered (Fig. 5F).

Discussion

As a result of their intrinsic properties, OECs and genetically modified OECs have been extensively transplanted into the injured spinal cord to promote axonal regeneration (Ramon-Cueto and Nieto-Sampedro, 1994; Li et al., 1997; Ramon-Cueto et al., 1998; Navarro et al., 1999; Ramon-Cueto et al., 2000; Ruitenberg et al., 2002; Garcia-Alias et al., 2004; Moreno-Flores et al., 2006). However, several studies failed to find unique migratory properties of OECs when implanted into damaged brain or lesioned spinal cord lesion (Gudino-Cabrera et al., 2000; Nieto-Sampedro, 2003; Lu et al., 2006; Pearse

et al., 2007). Although several authors addressed their attentions on OEC interaction with growing axons (Sorensen et al., 2008; Chuah et al., 2011), a number of parallel studies started to analyze the intrinsic migratory properties of OECs in order to improve their effectiveness after transplantation. Thus research into OEC migration and proliferation has recently been carried out on over glass surfaces with artificially generated gradients (Yan et al., 2003; Cao et al., 2006; Vukovic et al., 2009; Huang et al., 2011), in collagen scaffolds (Wang et al., 2006; Mollers et al., 2009), in nanofibers (Shen et al., 2010) and in biomaterial-coated substrates (Martin-Lopez et al., 2010b; Martin-Lopez et al., 2010a).

TROY (Morikawa et al., 2008) and NgR1 are expressed in OECs (Woodhall et al., 2003; Su et al., 2007). However, the present study is the first report that cultured rodent OECs express all the elements of the Nogo Receptor complex. In addition, we demonstrated that this receptor complex is active since OECs activate RhoK and increase ERK1-2 phosphorylation in response to Myelin. Indeed, MAIs inhibit OEC migration over grass surfaces but also in linearly elastic PAA gels.

From a biophysical point of view, our MSD data analysis indicates that cultured OECs migrate in an anti-persistent (subdiffusive) manner both over Laminin and Myelin in glass and PAA. In addition, our results may explain, to some extent, the observations of Gudiño-Cabrera and coworkers, who reported that transplanted OECs migrate preferentially in the opposite direction to the regenerating axon target and thus fail to promote axon regeneration (Gudino-Cabrera et al., 2000). In fact, OECs transplanted into the lesioned spinal cord must be placed at both sides of the lesion and close to it in order to improve effectiveness (Pearse et al., 2007). On the basis of our MSD data, we propose that additional factors are required to generate a persistent and directed movement of OECs *in vivo* (Lankford et al., 2008), as reported *in vitro* (Huang et al., 2008).

OECs show lower migratory potential in lesioned spinal cord compared to controls (Deng et al., 2006). In this regard, anti-NgR1-treated OECs show greater migration in spinal cord in white matter tracts than controls (Su et al., 2007). In fact, in the same study, OECs cultured on Nogo-66-coated coverslips showed increased adhesion and reduced migration as a result of greater formation of FAs and reduced membrane protrusions promoted by RhoA activation (Su et al., 2007). In our study, OECs cultured

on Myelin largely reduced the number of protrusions, stress fibers and FAs (Vinculin-positive), and these effects were partially reversed by the NEP1-40 peptide. These data also paralleled those obtained from PAA gels. The differences in FA dynamics between our data and those reported by Su and coworkers could be attributed to the use of a recombinant peptide instead of complete Myelin extract, respectively. Using TFM, we determined that increased traction force generation provides OECs cells with greater migratory capacity (on Laminin) and this capacity is reduced on Myelin-coated substrate. In addition, we observed a decrease in cell protrusions, disorganization of stress fibers and absence of FAs. The contribution of FAs to cell migration has been widely debated. In terms of traction forces, FA dynamics seems to play a dual role; first by increasing traction forces but later decreasing these forces, thereby, maintaining a cell spread morphology (Beningo et al., 2001). Su et al. (2007) reported that OECs growing on Nogo-66, resembled flat-sheet cells. This observation could be attributed to a maintenance role of FA with little migration potential, as suggested for aOECs. However, although we cannot rule out cell-specific factors for OECs, FA distribution and FA-mediated signaling in several cell types correlates with traction force parameters, nuclear translocation and cell migration (e.g. keratinocytes (Fournier et al., 2010), invasive tumor cells (Mierke et al., 2008) or fibroblasts (Wang et al., 2001; Li et al., 2005)).

In addition, our data show that OECs cultured on PAA substrates, the mechanical properties of which clearly differ from substrates coated on glass slides, increased their migratory potential since traction forces are generally enhanced between coated glasses and matrices. In this regard, recent data report increased proliferation and migration of OECs growing on several scaffolds (see above). Thus, it is feasible that a nanostructured scaffold would serve as a mechanical guide for OEC migration, thereby preventing the interaction of these cells with a putative inhibitory ECM. In this respect, migrating cells sense and respond to external stimuli. In fact, mechanical inputs can also be powerful regulators of cell behavior (Fournier et al., 2010). In physiological conditions, the migration of most cells occurs in an adhesion-dependent manner and involves the formation of FA at the membrane and the generation of mechanical forces via the actin-myosin network (Fournier et al., 2010). MAIs and receptors modulate the establishment of this network as well as microtubule dynamics (Schwab, 2010; Llorens et al., 2011). Indeed, MAI-induced arrest of CNS axon growth is either induced after MAI receptor, the Ca^{2+} -dependent activation of the epidermal growth factor receptor

(EGFr), and/or by the sequential Rho-A/ROCK/LIM-kinase/cofilin phosphorylation leading to actin depolymerization (Hsieh et al., 2006). In parallel, microtubule stabilization is also compromised after exposure to MAIs (Mimura et al., 2006). In our experiments, OECs in contact with Myelin reduced their protrusions and showed an almost uniform actin cytoplasmic labeling, which correlates with higher RhoA activation and ERK1-2 phosphorylation. The addition of the NEP1-40 peptide partially restored the migratory properties of OECs, in contrast to the full mechanical recovery observed when these cells were grown on heat-denatured Myelin. These observations indicate that other molecules present in Myelin extracts act on receptors other than the Nogo receptor complex and may modulate OEC migration. In this regard, new MAI receptors and ligands have recently been described (Schwab, 2010). Indeed, not only proteins but also Myelin-derived lipids may modulate axon growth and also cell migration (Thomas et al., 2010). Thus it is feasible that, in addition to unknown factors, some of these ligands/receptors may act together to modulate OEC migration.

In conclusion, our data suggest that a cell-based strategy using OECs that has the capacity to overcome the inhibitory action of MAIs is required in order to enhance the migratory properties and the persistence of OECs in axon regrowth and their functional recovery in the lesioned CNS.

Materials and methods

Antibodies and biochemical reagents

The following antibodies were used at a dilution of 1:1000 for Western blotting and/or 1:500 for immunohistochemical staining, unless otherwise indicated. Lingo-1 and Actin (dilution 1:10,000) were from Millipore (Billerica, MA, USA). S100 β was purchased from Abcam (Cambridge, MA, USA) and TROY from R&D System (Minneapolis, MN, USA). GFAP was from DAKO (Glostrup, Denmark), p75 was from Promega (Madison, WI, USA). NgR1 was a gift from Prof. B.L. Tang (Singapore). Tubulin (1:5000), Vinculin (1:400), Phalloidin-TRITC and DAPI were purchased from Sigma (St. Louis, MO, USA). ERK phospho-threonine 202/phospho-tyrosine 204 (pERK1-2) was from Cell Signaling Technology (Beverly, MA, USA). Total ERK antibody was from Transduction Laboratories (Lexington, KY, USA). Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit immunoglobulins were purchased from Molecular Probes (Leiden, Netherlands). The goat anti-mouse horseradish peroxidase (HRP) and rabbit anti-goat-HRP secondary antibodies used in the Western blots were from DAKO. Goat anti-rabbit-HRP was supplied by Sigma. Green fluorescent beads (0.75 μm \varnothing) used to bind Myelin were kindly provided by Dr. A. Homs and Prof. J. Samitier (IBEC, Barcelona). In addition, the Nogo-66 (1-40) antagonist peptide (NEP1-40) was purchased from Alpha Diagnostic International (San Antonio, TX, USA). Myelin was purified from adult Sprague-Dawley rat CNS, as described (Seira et al., 2010). All animal procedures were performed in accordance with the guidelines established by the Spanish Ministry of Science and Technology and the European Community Council Directive 86/609 EEC.

TEG3 cultures

The immortalized clonal cell line TEG3, which contains the SV40 large T antigen stable transfectant of OEG primary cultures, was prepared from adult rat olfactory bulbs (Moreno-Flores et al., 2003). Cells were maintained in ME10: DMEM-F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Bovine calf serum (SAFC Biosciences, Lanexa, VA, USA), 20 $\mu\text{g}/\text{ml}$ pituitary extract (Invitrogen), 2 μM forskolin (Sigma), 1% Penicillin-Streptomycin and 1% Fungizone (Invitrogen). TEG3 cells between passages 4 to 8 were used for the experiments.

Western blotting techniques

Cultured TEG3 cells were collected with a scraper and homogenized on ice in a buffer containing 150 mM NaCl, 50 mM HEPES, 1 mM ethylene-glycol-tetraacetic acid, 10% glycerol, 1% Triton X-100, and 13 protease inhibitor cocktail (Roche, Basel, Switzerland). The lysate was clarified by centrifugation at 12,000 *g* for 15 minutes, and the protein content of soluble fractions was determined using the Bio-Rad detergent-compatible assay (BCA) (Bio-Rad, Hercules, CA, USA). Cell extracts (20 µg) were boiled in Laemmli sample buffer at 100°C for 10 minutes, subjected to 8% or 15% SDS-PAGE, and electrotransferred to nitrocellulose membranes (Amersham Biosciences, United Kingdom). Extracts of mouse brain at postnatal day 5 were prepared and used as controls (see (Seira et al., 2010), for technical details). After transfer, membranes were incubated overnight with primary antibodies at 4°C. The following day, they were subsequently incubated with peroxidase-tagged secondary antibodies, and peroxidase activity was detected using the ECL-plus kit (Amersham Biosciences). Active RhoA was determined with a pull-down technique using the GST-Rhotekin-binding domain, as described previously (Ren et al., 1999), following the manufacturer's instructions (Rho Activation assay kit BK036, Tebu-Bio Barcelona, Spain) .

In vitro experiments and immunocytochemical methods

Glass coverslips (12 mm ø) were coated essentially as described (Fournier et al., 2001). Briefly, coverslips were precoated with poly-L-Lysine 10 µg/ml dissolved in 0.1 M PBS (pH 7.3) and then washed. They were then incubated with Myelin (0.01-0.02 µg/µl), heat-inactivated Myelin (0.02 µg/µl heated at 96°C for 1 hour) or 0.1 M PBS alone, and then allowed to dry. Next, coverslips were washed and coated with Laminin (2 µg/ml, dissolved in 0.1 M PBS) and washed again with 0.1 M PBS. TEG3 counted cells were seeded onto substrate-coated coverslips in ME10 medium. Some of the cells seeded on coverslips containing Myelin were treated with 1 µM NEP1-40 by adding the peptide directly to the culture media. Cells were cultured for 20 hours and then coverslips were fixed in 4% buffered paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1 M PBS, and blocked with 10% normal serum in 0.1 M PBS. Cells were sequentially incubated overnight with primary antibodies at 4°C and then with Alexa Fluor-tagged secondary antibodies for 1 hour. Cells were rinsed in 0.1 M PBS, stained with 0.1 µM DAPI diluted in 0.1 M PBS for 10 minutes, rinsed in 0.1 M PBS and mounted on Fluoromount™ (Vector Labs, Burlingame, CA, USA).

Adhesion stripe assays

Stripe assays were carried out as described previously (Nguyen-Ba-Charvet et al., 2001; Prestoz et al., 2004; Bribian et al., 2008). Briefly, acid washed coverslips were incubated overnight with poly-L-lysine (10 $\mu\text{g}/\text{ml}$; Sigma) at 37°C, and after were rinsed several times with water and air-dried. They were then, inverted onto a silicon matrix provided from Dr. J. Jung (Max Planck Institute, Tübingen, Germany). Myelin (0.02 $\mu\text{g}/\mu\text{l}$) or Laminin (2 $\mu\text{g}/\text{ml}$; Sigma) were mixed in 0.1 M PBS and the stripes were performed. The first protein was injected into the matrix channels and incubated for 2 hours at 37°C. A solution of fluorescent Alexa 594-conjugated bovine serum albumin (2% in Laminin; Molecular Probes) was then injected into the channels. After 2 hours at 37°C, the channels were rinsed four times by injecting 0.1 M PBS. TEG3 cells were placed on the stripes and cultured for 24 hours as indicated above. Stripe-functionalized coverslips were then fixed with 4% buffered paraformaldehyde for 10 minutes before DAPI staining, mounted in mounted on Fluoromount™ and photo-documented in an Olympus BX61 fluorescence microscope.

Time-lapse analysis of TEG3 migration

Fluorodish cell culture dishes (World precision instruments, Sarasota, FL, USA) were coated with Laminin or Myelin, as described above. We seeded 5×10^4 TEG3 cells on the coated dishes and 20-24 hours later we performed the time-lapse analysis. To study cell migration, we transferred the culture dishes to an LCI system (Live Cell Instruments, Seoul, Korea) for 20 hours. The multi-tracking analysis was performed with the ImageJ™ software using the plugin mTrackJ (Biomedical Imaging Group Rotterdam of the Erasmus MC-University Medical Center Rotterdam, Netherlands). Tracking was performed in an inverted Olympus microscope IX-71 (20X objective) and the images (5 megapixels) were captured by an Olympus XC-50 camera (150 frames, 1 frame every 8 minutes). Cell tracking allows the analysis of the scrolling speed and position frame, (Xt, Yt). The cell position for each frame (position pixel) was determined with Matlab™ (MathWorks, Massachusetts, MA, USA), which provides a 3D graphic (x-y-z, z = time) and the MSD. The same experiments were run over Fluorodishes covered with ~70-100 μm thick PAA gels prepared as previously described (Dembo and Wang, 1999). Briefly, to obtain a stiff gel of 12 kPa Young Modulus (PAA), 265 μl of an Acrylamide/Bis-acrylamide mixture (15% Acrylamide and 6.5% Bis-acrylamide, Bio-Rad) was dissolved in ultrapure water containing 0.4% of 0.2 μm diameter red fluorescent beads (Invitrogen), 0.5% ammonia persulfate and 0.05% TEMED (Bio-Rad). For multi-tracking, 3D plot and MSD of the TEG3

migration, the mixture was added to the center of the dish, which was then coated and stored overnight at 4°C. Previous to this procedure, OEC adhesion analyses were performed using 12 kPa, 1.4 kPa and 0.15 kPa PAA gels. 12 kPa PAA gels were selected since OECs show greater adherence and migration in these gels.

Traction force measurement

Cell tractions were evaluated using constrained Fourier transform traction microscopy (FTTM) (Butler et al., 2002). Briefly, the displacement field was calculated by comparing fluorescent microbead images obtained during the experiment with a reference image taken at the end of the experiment after the trypsinization and the consequent detachment of OECs from the underlying substrate. The projected cell area was calculated with MatlabTM, based on the manual tracing of the OEC contours determined by a phase contrast image obtained at the start of the experiment. A particle imaging velocimetry algorithm (Trepate et al., 2009) was used to determine the deformation of the substrate caused by the traction forces.

Statistical analysis

Summary data are expressed as mean \pm s.e.m. (standard error of the mean) of at least three independent experiments. Means were compared by one-way ANOVA test. A $P < 0.05$ was considered statistically significant.

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