Generating Oligodendrocyte Progenitor Cells and Measuring their Dielectric Properties

Chesca A. Barrios
Biological Sciences

Author

Chesca Barrios started her research through the UCI STEM Summer Bridge Program as a student at Mt. San Antonio College. She continued work on her project after transferring to UC Irvine and remained in Dr. Flanagan's lab until her graduation in spring 2012. For Chesca, the most rewarding part of her research has been the opportunity to make new discoveries that can potentially help other researchers and, eventually, the public. She hopes to attend medical school and believes her undergraduate research experience has helped her develop discipline and a strong work ethic that will be critical tools for her continuing education and future career.

Abstract

Dielectrophoresis (DEP) is a novel way of sorting and characterizing neural stem cells and their progeny. Our group has found that a neural stem cell's dielectric property—whether it traps at a specific frequency range or not—reflects cell fate bias and can be used to identify its specific progenitor cells. Although DEP has been applied to neuron and astrocyte progenitor cells, it has yet to be used for oligodendrocyte progenitor cells (OPCs). The purpose of this project is twofold: to generate OPCs and confirm their presence through immunostaining and cell migration, and to use DEP to determine OPCs' dielectric properties. This characterization is increasingly significant because of the field's recent success in treating neurological diseases and injuries with transplanted OPCs. Migratory behavior and expression of platelet-derived growth factor (PDGF) receptor-α (PDGFR-α) confirmed the OPC phenotype while O4 expression confirmed differentiation of OPCs into oligodendrocytes. Moreover, the DEP trapping curve of OPCs is significantly different from that of heterogeneous neural stem progenitor cell controls (NSPCs) derived from the same starting cells. This dielectric signature of OPCs is closer to that of neuron progenitor cells (NPCs), which could provide an insight to the development of the OPC lineage.

Key Terms

- Dielectrophoresis
- Neural Stem Cells
- Oligodendrocytes

Faculty Mentor

Neural stem cells generate specialized progenitors biased to form one of the final differentiated cells of the brain and spinal cord. These cells have enormous potential for the treatment of neurological disease and injury. However, little is known about the properties that distinguish the progenitors in this lineage. Chesca Barrios addressed this by establishing protocols in my lab to culture oligodendrocyte progenitor cells and measure their plasma membrane properties. She identified novel characteristics of these cells that distinguish them from other progenitors. These studies help to clarify the cell biological differences controlling the formation of the different types of mature central nervous system cells. Chesca's successful research demonstrates the vital role committed and hard-working undergraduates can play in scientific discovery.

Lisa Flanagan
School of Medicine

Introduction

Stem cells hold therapeutic promise because they are pluripotent and can differentiate into any type of cell, thus providing cellular repair during disease or injury. Neural stem cells of the central nervous system, in particular, form specific progenitor cells before generating differentiated neurons, astrocytes, or oligodendrocytes. Cultures of neural stem cells include undifferentiated stem cells and more differentiated progenitors of all three types. Because of the tendency to produce heterogeneous populations, isolating specific progenitors for characterization or transplantation has been challenging. Specific binding of antibodies to an antigen indicates the presence of a cell surface marker and can be used to distinguish specific progenitor populations. For instance, the cell surface antigen A2B5 has been proposed as a marker for astrocyte progenitors (Rao et al., 1998). However, other studies claim that A2B5 is also expressed by both neuronal and oligodendrocyte progenitors (Xia et al., 2003). Currently, there are no cell surface markers that can exclusively distinguish a specific progenitor population from heterogenous neural stem cells. Isolating progenitor cells is important because purer populations can provide greater control over the differentiated phenotype of transplanted cells. For instance, where it may be desired to form new neurons for treating central nervous system injuries, transplanted neural stem cells receive signals from the damaged tissue inducing them to form astrocytes. If a pure population of neuron restricted progenitor cells is transplanted instead, this predicament could potentially be avoided.

Dielectrophoresis (DEP) is a novel and marker-free way to distinguish stem cells from their more differentiated progeny and could be used to purify a particular neural progenitor cell population (Flanagan et al., 2008). DEP involves the application of a non-uniform electric field that induces a frequency-dependent dipole in cells. The DEP force selectively traps cells along electrodes depending on the cell's dielectric properties, which include the plasma membrane conductance and capacitance. Previous applications of DEP include separating leukemia cancer cells out of the blood, and malaria-infected red blood cells from normal cells (Altomare et al., 2003). In the same vein, neuronal progenitor cells and astrocyte progenitor cells were found to have unique dielectric properties that reflect their respective cell fate biases (Flanagan et al., 2008). Further work has shown that exposure of neural stem and progenitor cells to DEP electric fields for the short times necessary for sorting does not damage the cells, change their ability to divide, or affect their differentiation (Lu et al., pers. comm.). Thus, DEP may be useful for isolating a specific population of progenitor cells, which can then be used for transplantation to treat injuries or diseases of the central nervous system.

Oligodendrocytes and their progenitor cells have yet to be characterized using DEP. Oligodendrocytes wrap myelin around neurons for faster conduction of action potentials and better axon survival. Characterizing oligodendrocyte progenitor cells (OPCs) is increasingly significant because of recent advances in stem cell therapy. For instance, OPCs transplanted in rat spinal cord injuries have been shown to remyelinate neurons and restore locomotion (Kierstead et al., 2005). Although it is known that neuron progenitors and astrocyte progenitors give rise to neurons and astrocytes, respectively, the specific lineage of progenitors leading to oligodendrocytes is still debatable. Some published reports trace the oligodendrocyte lineage to NG2-glia cells, which give rise to oligodendrocytes predominantly, but also produce astrocytes (Tripathi et al., 2010). Others suggest that OPCs can have alternative fates and could go through a neurogenic lineage (Liu et al., 2007).

The purpose of this project was twofold: OPCs were first generated and their phenotype confirmed, and then their dielectric properties were measured for future optimization of the isolation of the OPC lineage. Moreover, since neurogenic and astrogenic dielectric properties had already been characterized, the OPC dielectric signature could be compared with these, and thus shed light on OPCs' lineage, since progenitors sharing a common lineage may have similar dielectric properties.

Materials and Methods

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with Federal guidelines. The procedure is illustrated in Figure 1.

Figure 1

Cell Culture

Cells were isolated from the cerebral cortical regions of wildtype CD1 mice at embryonic day E16.5. The cells were divided into two groups: one expanded as a heterogenous population of neural stem progenitor cells (NSPCs), and the other cultured in conditions that encourage the generation of OPCs. NSPCs, which served as the control for subsequent experiments, were grown as neurospheres in Temple media enriched with 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor

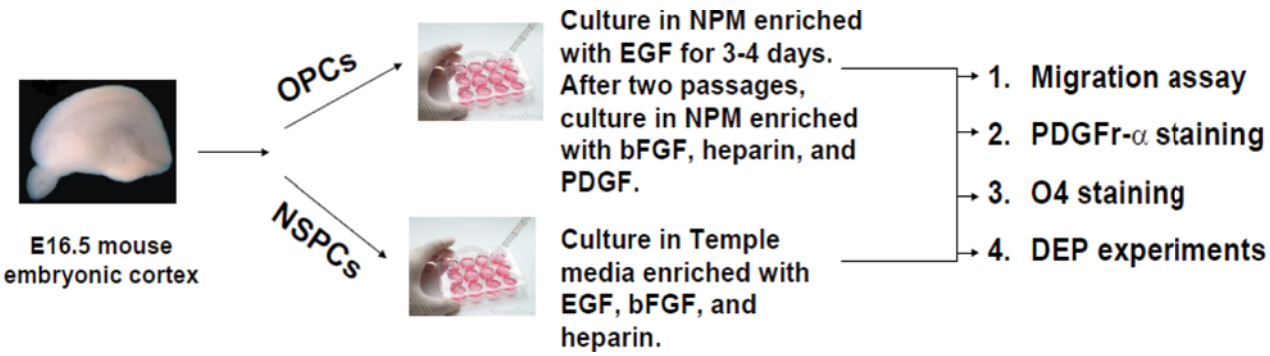

Figure 1
Summary of Procedure

(EGF), and 20 ng/mL heparin (Flanagan et al.,2008). NSPC neurospheres were passaged every three to four days using Neurocult dissociation buffer. To generate OPCs, a protocol from *Pedraza et al.* was used with some modifications. Cells were grown as neurospheres in neural proliferation media (NPM) consisting of Dulbeco's Modified Enriched Medium/F1 and B27 with 20 ng/mL EGF (Pedraza et al., 2008). After three to four days, the neurospheres were dissociated using Neurocult dissociation buffer and resuspended in NPM with EGF. After a second passage into NPM with EGF, neurospheres were dissociated for the third passage and resuspended in NPM with the following growth factors: 20 ng/mL bFGF, 20 ng/mL heparin, and 20 ng/mL platelet-derived growth factor (PDGF). These OPC neurospheres were passaged every four to five days. For both NSPCs and OPCs, passages two to six were used for experiments.

For cell differentiation, NSPC neurospheres were dissociated and plated on 12 mm laminin- coated coverslips. Since growth factor withdrawal facilitates differentiation, cells were kept in Temple without growth factors for a total of five days. OPCs were also dissociated and plated on 12-mm laminin-coated coverslips. Cells were kept in NPM without any growth factor for two days and were then treated with 20 ng/mL ciliary neurotrophic factor (CNTF) for seven days.

Cell Migration

In order to monitor and compare cell migration patterns, NSPC and OPC spheres were plated on 12-mm laminin-coated coverslips and were imaged using an EVOS microscope after 1.5 hours. Migration patterns were analyzed by counting the percentage of spheres that display migratory behavior, with individual cells distinctly emanating from the plated sphere.

Immunostaining

Two days after plating, undifferentiated cells were fixed with 4% paraformaldehyde and immunostained as previously described (Flanagan et al., 2007) for the expression of PDGFR-α (1:200 dilution, Genetex). Differentiated cells were fixed with 4% paraformaldehyde and stained for the expression of O4 (1:200, R&D Systems), a mature oligodendrocyte cell surface marker. Since PDGFR-α and O4 are cell surface markers, detergent was not used for permeabilization in the immunostaining protocol. Fluorescent images of cells were taken using a Nikon Ti-E microscope, merged using Adobe Photoshop, and analyzed using ImageJ counting software.

DEP Trapping Curves

For the DEP experiments, both OPC and NSPC neurospheres were dissociated using Neurocult dissociation buffer and resuspended in DEP buffer (8.5% sucrose [wt/vol], 0.3% glucose [wt/vol], and 0.725% [vol/vol] RPMI) to a concentration of 1 x 106 cells/mL. The DEP buffer used for each experiment had a conductivity value between 105.3-110.4 μS/cm. The experimental setup consisted of a DEP device that was placed on the stage of an upright microscope and connected to a syringe pump to control fluid flow (Flanagan et al., 2008). The electrodes were connected to a function generator to produce the DEP force. Cells were loaded in the device and then monitored using the upright microscope and color camera connected to a computer.

To ensure proper cell trapping, 70% ethanol, water, 5% bovine serum albumin, and DEP buffer were passed sequentially through the device before each experiment. This preparation sterilized the device and prevented non-specific cell adhesion to the electrodes. Cells were then loaded to the device with a fluid flow of 1.5 μL/min. The function generator was set at an amplitude of 8 Vpp while the frequency varied from 25 Hz to 20 MHz. A five-second video was

recorded for each frequency and the videos were later quantified to determine the percentage of cells trapped at each DEP frequency. Student's *t* test was used for data analysis of three independent experiments, and $p < 0.05$ was considered significant. The percentage values were expressed as means +/- the standard error of mean, and the graphs were normalized using the highest mean of a data set.

Results

Because the two sets of cells, cultured as NSPCs and OPCs, were isolated from the same E16.5 embryonic cortex, the heterogeneous NSPCs could serve as a control for the OPCs in order to confirm their phenotype. Migratory behavior of OPCs has been suggested as a way to distinguish the OPC lineage from other progenitor populations (Pedraza et al., 2008). Cell migration was assayed by plating NSPC and OPC spheres and observing how cells migrated out of the spheres over time. Images taken of NSPC spheres show that they do not display visible migratory behavior 1.5 hours after plating (Figure 2A). On the other hand, 100% of plated OPC spheres displayed a distinct migratory pattern (Figure 2B).

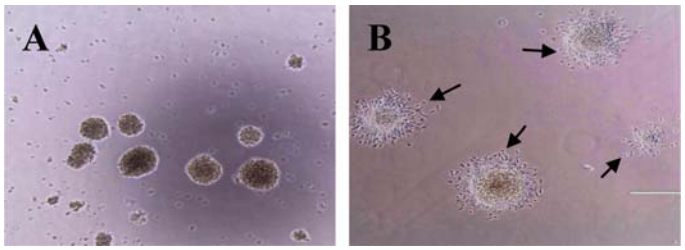

Figure 2

OPCs are more migratory than NSPCs. NSPC spheres imaged after 1.5 hours did not display any migratory behavior (A). OPC spheres imaged after 1.5 hours exhibited cells that are distinctly emanating from the spheres, as represented by the black arrows (B).

PDGFR-α is expressed by cells in the early stages of OPC development (Ellison, 1994). To further confirm the OPC phenotype and distinguish it from the NSPC control, both OPCs and NSPCs were immunostained for the expression of PDGFR-α. NSPCs did not express PDGFR-α after two weeks in Temple media (Figures 3A and C). In contrast, OPCs in NPM were approximately 85% PDGFR-α positive (Figures 3B and D). Moreover, 100% of cells in an OPC sphere exhibiting migratory behavior stained positive for PDGFR-α (Figure 3D).

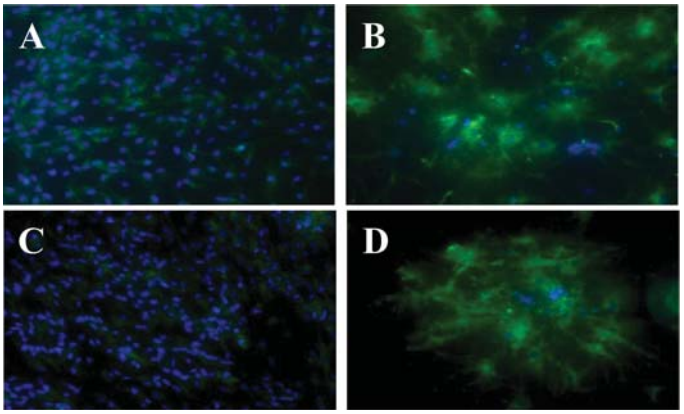

Figure 3

OPCs expressed PDGFR-α while NSPCs did not. Cells were immunostained for the following markers: PDGFR-α (cell surface protein, green) and Hoechst (nucleus, blue). NSPCs did not express PDGFR-α (A and C) while OPCs did (B and D). Moreover, an OPC sphere displaying migratory behavior is 100% positive for PDGFR-α (D).

O4 expression marks the beginning of preoligodendrocyte maturation and is expressed by both immature and mature oligodendrocytes (Sommer, 1981). Hence, OPCs would be expected to differentiate into oligodendrocytes and acquire the O4 marker. To further confirm OPC production and distinguish these cells from the NSPC control, both sets of cells were differentiated and stained for the O4 marker. NSPCs differentiated for five days barely expressed O4 (0.07%), while OPCs treated with CNTF had a slightly higher expression (2.9%), and exhibited the morphology of mature oligodendrocytes (Figure 4).

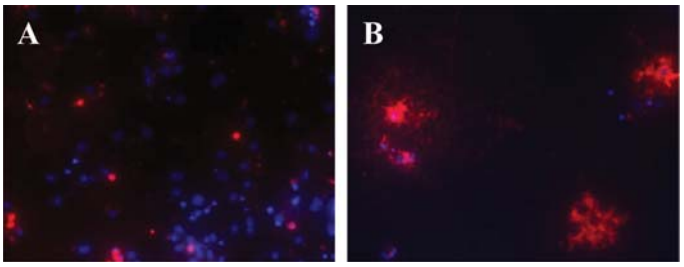

Figure 4

OPCs differentiated into more oligodendrocytes than NSPCs. Cells are immunostained for the following markers: O4 (cell surface protein, red) and Hoechst (nucleus, blue). NSPCs had a very low O4 expression (A). OPCs have a slightly higher O4 expression and display the cell morphologies of mature oligodendrocytes (B).

The OPCs generated expressed PDGFR-α, had a distinct migratory capacity, and generated oligodendrocytes better than NSPCs did. To further characterize these OPCs, their dielectric properties were measured by using DEP frequen-

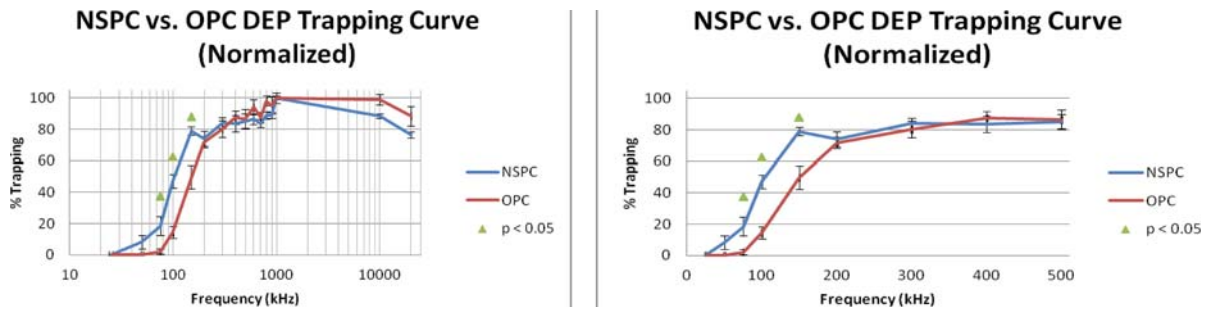

Figure 5

The OPC trapping curve is statistically different from the NSPC trapping curve. The steepest part of the OPC trapping curve is shifted to the right compared to the NSPC control (left panel). The shift occurs within the statistically significant frequency range of 75 kHz to 300 kHz (right panel); n=3 independent experiments, error bars are s.e.m.

cies to determine a DEP trapping curve (Flanagan et al., 2008). The DEP trapping curve of OPCs is shifted to the right in the 75 kHz to 300 kHz frequency range, compared to the NSPC trapping curve (Figure 5). This is corroborated by the statistical significance of the same frequency range. Using a Student's *t* test, the percentage of cell trapping for the following frequencies were highly significant ($p<0.05$): 75 kHz ($p=0.0162$), 100 kHz ($p=0.0000346$), and 150 kHz ($p=0.0000347$). DEP experiments (n=3) thus confirm the hypothesis that OPCs have dielectric properties that distinguish them from NSPCs.

Discussion

We sought to determine the dielectric properties of OPCs in order to optimize the isolation of this lineage by DEP. OPCs were generated and their phenotype confirmed by comparing them to control heterogeneous NSPCs isolated from the same starting material. Since OPCs migrate toward neurons in order to perform their myelinating function, cell migration has been used to confirm the presence of OPCs (Ono et al., 1997). Cell migration assays revealed that the generated OPCs were more migratory than NSPCs. This is consistent with published reports stating that cells grown in PDGF and bFGF generate more abundant migratory OPC-like cells, compared to those plated solely in either bFGF, EGF, or PDGF (Pedraza et al., 2008). This suggests that PDGF, in conjunction with bFGF, induces NSPCs to a highly motile oligodendrocyte lineage (Pedraza et al., 2008).

The delayed maturation of gray matter oligodendrocytes is marked by high levels of PDGFR-α expression, while the early maturation of white matter oligodendrocytes reflects lower levels (Ellison, 1994). This has established PDGFR-α as a marker for the early stages of oligodendrocyte development. The OPCs generated in this study predominantly expressed PDGFR-α, while NSPCs did not express the marker at all. This suggests that the OPCs generated are in the early stages of the OPC lineage, and that they are distinct from the control cells.

After the migration of OPCs in the central nervous system, they settle in the future white matter of the brain as preoligodendrocytes (Sommer, 1981). At this stage, PDGF receptors disappear and another cell surface molecule, O4, is expressed instead (Ellison, 1994). Because O4 expression persists from the preoligodendrocyte stage to mature oligodendrocyte differentiation, its expression can be used to confirm that the generated cells can be induced to differentiate as oligodendrocytes. The NSPC control had a very low expression for O4, while OPCs had a slightly higher expression; however, O4 positive cells derived from OPCs had a more mature oligodendrocyte morphology not observed in NSPCs. Thus, because the OPCs have abundant migratory behavior and predominant PDGFR-α expression, and can be induced to differentiate as mature oligodendrocytes, their OPC phenotype can be confirmed. The relatively low O4 expression after OPC differentiation may be due to specific molecular signals and microenvironment absent in the *in vitro* experiments. For instance, previous studies found that hypoxic (low oxygen) conditions were required to generate oligodendrocytes *in vitro* (Pistollato et al., 2007). Future work may involve differentiating both OPCs and NSPCs in hypoxic conditions to check if this increases O4 expression and oligodendrocyte differentiation.

OPCs were further characterized by measuring their dielectric properties. Comparing the DEP trapping curve of OPCs with that of NSPCs showed a significant difference in the percentage of cell trapping. In particular, the following frequencies, which correspond to the steepest part of the curve, showed the greatest difference: 75 kHz ($p=0.0162$), 100 kHz ($p=0.0000346$), and 150 kHz ($p=0.0000347$). The rightward shift of the curve indicates that the percentage

of trapped NSPCs is greater than that of OPCs in lower frequencies. Previous studies found that neuron progenitor cells trap at higher frequencies while astrocyte progenitor cells trap at lower frequencies (Flanagan et al., 2008). The OPC trapping curve occurs at frequencies similar to those of neuron progenitors. Thus, the OPC dielectric signature is closer to that of neuron progenitor cells, which could suggest that the OPC lineage is closer to the neuron progenitor lineage as opposed to the astrocyte progenitor lineage. Moreover, a cell's membrane capacitance, which affects how it responds to the DEP force, has been found to correlate directly with astrogenic potential and inversely with neurogenic potential (Lu et al., 2011).

Future work will include measurement of OPC membrane capacitance to determine whether it is similar to that of neuron or astrocyte progenitors. Furthermore, differentiation experiments will test whether the PDGFR-α positive cells also generate neurons or astrocytes, which could suggest a shared progenitor. The generation of OPCs and determination of their dielectric properties point to the possibility of isolating purified OPC populations by DEP. This can be used to optimize OPCs for transplant in treating neurological diseases and injuries. This study also sheds light on OPC lineage and enhances our understanding of central nervous system development.

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