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Short Report: Olfactory Ensheathing Cells Promote Differentiation of Neural Stem Cells and Robust Neurite Extension

Rosh Sethi[$],
Harvard Medical School, 25 Shattuck Street, Boston MA 02115

Roshan Sethi[$],
Harvard Medical School, 25 Shattuck Street, Boston MA 02115

Andy Redmond, and
Precision Spine Care,1814 Roseland Blvd, Suite 200, Tyler, TX, 75701

Erin Lavik
Biomedical Engineering, Case Western Reserve University, 10900 Euclid Ave., phone: 216-368-0400

Abstract

Aims—The goal of this study was to gain insight into the signaling between olfactory ensheathing cells (OECs) and neural stem cells (NSCs). We sought to understand the impact of OECs on NSC differentiation and neurite extension and to begin to elucidate the factors involved in these interactions to provide new targets for therapeutic interventions.

Materials and Methods—We utilized lines of OECs that have been extremely well characterized in vitro and in vivo along with well studied NSCs in gels to determine the impact of the coculture in three dimensions. To further elucidate the signaling, we used conditioned media from the OECs as well as fractioned components on NSCs to determine the molecular weight range of the soluble factors that was most responsible for the NSC behavior.

Results—We found that the coculture of NSCs and OECs led to robust NSC differentiation and extremely long neural processes not usually seen with NSCs in three dimensional gels in vitro. Through culture of NSCs with fractioned OEC media, we determined that molecules larger than 30 kDa have the greatest impact on the NSC behavior.

Conclusions—Overall, our findings suggest that cocultures of NSCs and OECs may be a novel combination therapy for neural injuries including spinal cord injury (SCI). Furthermore, we have identified a class of molecules which plays a substantial role in the behavior that provides new targets for investigating pharmacological therapies.

Correspondence to: Erin Lavik.

[$]contributed equally

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Keywords

CNS injury; olfactory ensheathing glia; neural progenitor; tissue engineering; scaffold; paracrine signaling

Introduction

One of the holy grails in stem cell biology is to successfully terminally differentiate stem cells or progenitors for therapies. There has been a tremendous amount of excellent work along these lines [1–4]. However, it has been extremely challenging to direct the differentiation of stem cells rapidly and in large numbers. Biology does this exceptionally well, and we asked whether we could begin to gain insight into the factors that biology uses through a simple coculture system of neural stem cells (NSCs) and olfactory ensheathing cells (OECs).

These cell types are particularly attractive for cellular therapies in the central nervous system, and a number of papers have investigated them following spinal cord injury (SCI). The environment of the injured spinal cord is inhospitable to recovery: healthy, mature neurons are incapable of dividing to replace injured ones, severed axons exhibit limited sprouting and growth, a glial scar forms which inhibits regeneration, and existing axons are often demyelinated [5–8]. Current treatment is limited to the use of high dose steroids, conservative management and rehabilitation.

OECs are a type of glial cell found in the lamina propria, a layer of tissue directly adjacent to the olfactory epithelium [8] and the olfactory bulb [9–12]. The olfactory epithelium is unique in the peripheral nervous system because it hosts the periodic neurogenesis of olfactory receptor neurons (ORNs) which are often damaged through the binding of odorants. ORNs are regenerated from a putative stem cell layer in the epithelium [13]. OECs are thought to support the regrowth of axons from nascent ORNs in the epithelium across the lamina propria and the cribriform plate to synapse in the olfactory bulb. OECs act through direct contact, by ensheathing dozens of emerging axons and extending sheet-like processes, and through the release of neurotrophic and adhesion factors [14]. Numerous studies have implanted these cells at the site of a spinal cord lesion to encourage the axonal regeneration following injury and have been shown to promote limited functional recovery in animal models [15–17] and recently in humans [18]. There is some evidence this recovery is achieved by the interaction of OECs with host astrocytic processes to build a bridge supporting nerve growth across the site of injury [19]. These effects have also been attributed to the secretion of neurotrophic growth factors, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which create a hospitable environment for axonal regeneration and elongation [8,20].

Neural stem cells (NSCs) represent another potential cell therapy for spinal cord injury. NSCs are similar to adult neural stem cells; they can differentiate into a limited number of lineages, including astrocytes, neurons and oligodendrocytes [21]. A variety of known soluble factors can provoke development down each of these lines [21]. NSCs are found in the developing and adult nervous system in several regions including the subventricular zone of

the lateral ventricles, the basal layer of the olfactory epithelium, the subgranular zone of the hippocampus [22] and the spinal cord [23]. They are cultured in aggregates of cells called neurospheres, a mixture of differentiated and undifferentiated cells. Because of their potential for differentiating into mature neural tissue, NSCs have been used in rat spinal cord injury implants and can promote functional recovery [24–26]. While there is evidence of NSC survival and proliferation after transplantation [27–29], there have been mixed reports on successful differentiation. Investigations with human NSCs in rat spinal cord injury show differentiation into neuronal lineages and even the development of synapses with local host cells [30,31], while similar studies suggest that differentiation is limited to only the astrocytic phenotype and may contribute to the glial scar [32,33]. Others have found that NSCs exhibit decreased survival when injected into injured spinal cord models [25].

Both OECs and NSCs exhibit some efficacy as cellular therapies for neurological insults and diseases. There have been a series of investigations in recent years looking at the coculture of NSCs and OECs. OECs have been shown to improve survival of dopaminergic neurons derived from NSCs in Parkinsonian rats [34]. Co-transplanation of NSCs with OECs has been investigated in a model of cognitive dysfunction showing better survival of cholinergic neurons from the NSCs in the presence of OECs [35]. Coculture of OECs and NSCs in an RGD-peptide modified gellan gum have been shown to lead to significantly improved survival and differentiation of the NSCs [36]. These cocultures have also been shown to improve function in rat models of spinal cord injury [37,38]. There is clear promise with using the two cell types together, and this has motivated our interest in investigating their interaction.

Here we describe an *in vitro* study investigating the effect of direct and indirect contact between OECs and NSCs. Using three dimensional co-cultures of NSCs and OECs in growth-factor reduced MATRIGEL ® matrix and two dimensional co-cultures of NSCs in OEC conditioned media (OEC CM), we demonstrate that OECs can significantly inhibit the proliferation yet promote the differentiation, migration, neurite outgrowth and maturation of NSCs in seven days. We report that that these effects are mediated by soluble factors, released by OECs, greater than 30 kDa in size. These results have implications for spinal cord injury therapy.

Materials and Methods

Two cell types were used in this work. One is a cell line that has been developed and published on previously, Immortalized adult rat olfactory ensheathing cells (Cell line # robp30a56 isolated by J. Silver according to methods described previously [39]). The second cell type, neural stem cells, was isolated from postnatal day 1 transgenic mice expressing green fluorescent protein (GFP) (isolation performed according to methods described previously [40–42]). All animal procedures followed the NIH guidelines for animal care and safety and were approved by the Animal Care and Use Committee of Yale University.

Isolation and maintenance of olfactory ensheathing cells

Immortalized adult rat olfactory ensheathing cells (Cell line # robp30a56 isolated by J. Silver according to methods described previously [39]). Briefly, olfactory bulbs were isolated

from Sprague-Dawley rats. After removal of the meninges and attached nerve, the tissue was minced in oxygenated SMEM (pH 7.0–7.4 (Gibco, Grand Island, NY). 1.0 mg collagenase (Sigma Type V), 0.4 mg/DNAse, 15 units papain (Sigma), and 1.0 mg Dispase-Collagenase (Boehringer Mannheim, Indianapolis, IN) was added followed by sieving and centrifugation. Cells were kept in DMEM, 10% FBS, 1% methyl cellulose, and 25–50% conditioned medium. Media was changed every 3 days. Within the first week of culture, the cells were immortalized with the SV40 large T antigen.

This cell line was cultured in T75 (cm^2) flasks in DMEM containing 10% fetal bovine serum, 5% heat inactivated calf serum and 1% penicillin/streptomycin/fungizone. Adherent cells were passaged (1:10) every 2 weeks.

Isolation and maintenance of neural progenitor cells

Neural stem cells were isolated from postnatal day 1 transgenic mice expressing green fluorescent protein (GFP) (isolation performed according to methods described previously [40–42]). Briefly, whole brains were removed from postnatal day 1 transgenic mice. The meninges were removed, and the remaining tissue was minced in then digested with 0.1% collagenase in PBS on ice. Cells were passed through a 100 um strainer and kept in cell culture media. After 3 days, neurospheres were separated and cultured. All animal procedures followed the NIH guidelines for animal care and safety and were approved by the Animal Care and Use Committee of Yale University. Following isolation, cells (which form neurospheres) were maintained in cell culture suspension in DMEM F12 containing epidermal growth factor, N2 supplement, B27 supplement, 1% penicillin/streptomycin/fungizone and L-glutamine in T75 (cm^2) flasks and passaged (1:2) every 2 weeks.

Olfactory ensheathing cell conditioned media library

Immortalized rat olfactory ensheathing cells (isolated by J. Silver according to methods described previously [39]) were thawed and cultured in T75 (cm^2) flasks in DMEM containing 10% fetal bovine serum, 5% heat inactivated calf serum and 1% penicillin/streptomycin/fungizone. Cell cultures were maintained for two days after fourth passage (1:4) and subsequently 10mL of medium was collected. Collected medium was centrifuged at 800rpm for 5 minutes to remove cell debris, and filtered with 0.2μm syringe driver filter unit (Millipore). Conditioned medium was stored at −20°C.

Olfactory ensheathing cell conditioned media fractionation

Isolated OEC conditioned media was fractioned via centrifugation. Filtration tubes (Millipore) with 10kDa, 30kDa and 100kDa molecular weight pores were hydrated with phosphate buffered saline (PBS) and subsequently centrifuged at 3000rpm for 5 minutes at 4°C. Unfractioned OEC conditioned media was added to each tube and centrifuged at 3000rpm for 30 minutes at 4°C. Filtrate from 10kDa tube (protein <10kDa) was collected and stored at −20°C. The concentrated protein residue (>10kDa) from this filtration was diluted in 5mL OEC media and added to a new 30kDa filtration tube. Filtrate from the 30kDa tube (10–30 kDa) was stored at −20°C and concentrated protein residue (>30kDa) was diluted in 5mL OEC media and added to a 100kDa filter tube. The filtrate (30–100 kDa) was collected and stored at −20°C. Filtrate from the 100kDa tube was discarded, and the

concentrated protein (> 100kDa) residue was stored at −20°C. A total of four fractions were collected: 0–10kDa, 10–30kDa, 30–100kDa and >100kDa.

3D in vitro coculture of NSCs and OECs

NSCs were co-cultured with OECs in growth-factor reduced MATRIGEL ® matrix (BD Biosciences). Frozen matrix was thawed and added to 8-well chambered borosilicate coverglass (NUNC) on ice at 150μL/cm^2 of well surface. 10,000 NSCs, obtained by trypsinizing neurospheres with 25% trypsin EDTA 1× (Sigma) and counted using hemacytometer, were seeded in gel by gently triturating cells in thawed MATRIGEL ® using cooled pipette tips followed by addition of appropriate number of OECs. OECs and NSCs were seeded in three different ratios: 1:1, 2:1, 5:1 (OEC:NSC). Control gels were seeded with 10,000 NSCs alone. Serum-free media was added to each well and chamber was incubated at 37°C with 5% CO_2 for 17 days. Immunocytochemistry analysis of gels was conducted in-well using immunocytochemistry protocol outlined below.

2D in vitro coculture of NSCs and OEC conditioned media

Sixth to eight passage neurospheres grown in suspension were removed from T75 (cm^2) flasks and centrifuged at 800rpm for 5 minutes. Supernatant was removed, and spheres were resuspended in 1mL of neural progenitor cell media. 50μL of suspension was removed, transferred to a microcentrifuge tube, and triturated using pipette to break up spheres into single cells. Cells were counted using hemacytometer. Volume of whole neurospheres equivalent to volume of 20 million cells was transferred to 12mm diameter poly-D-lysine/laminin coated glass discs (BD Biosciences) in 24 well plates (Costar), and subsequently incubated for 30 minutes in sterile incubator to promote cell adhesion. 750μL of OEC CM, OEC media (control, unconditioned), or neural progenitor cell media (control) was then added to wells. 24 well plates were kept in the 37°C incubator for 3 days or 7 days.

2D in vitro coculture of NSCs and fractioned OEC conditioned media

As in previously described co-culture assay, NSCs were counted and seeded on poly-D-lysine/laminin coated glass coverslips and incubated for 30 minutes at 37°C. Prior to adding fractioned, conditioned OEC media, fraction protein concentration was measured using Bio-RAD protein assay. Fractions were diluted such that 1.6mg/mL protein / fraction was added to each well. 24 well plates were incubated at 37°C for 7 days.

Immunocytochemistry analysis

NSCs on 12mm poly-D-lysine/laminin coated coverslips were fixed with 10% Formalin for 30 minutes after three or seven days. Spheres were blocked with 3% normal goat serum (NGS) in 0.3% TritonX-100 in phosphate buffered saline for 1 hour. Spheres were subsequently incubated with primary antibodies: rabbit anti-GFAP (Sigma, 1:160), rabbit anti-neurofilament (Sigma, 1:80), mouse anti-nestin (Sigma, 1:200), and mouse anti-β III tubulin (Sigma, 1:1000), mouse anti-GABA (Sigma, 1:2000), rabbit anti-tyrosine hydroxylase (Chemicon, 1:1000), goat anti-ChAT (Chemicon, 1:100), mouse anti-GAD 65/67 (Sigma, 1:1000), rabbit anti-glutamate (Sigma, 1:4000) and rabbit anti-Synapsin I (Sigma, 1:500) overnight. Antibodies were labeled with Alexafluor 647 (anti-Rabbit and

anti-mouse, Molecular Probes, 1:200) secondary antibody and visualized using Zeiss Axiovert microscope using Cy5 channel. Images were obtained using 20× objective and 5 images were procured per neurosphere (only cells on periphery of neurosphere were imaged). Immunocytochemistry analysis for 3D coculture in MATRIGEL ® was performed using similar protocol with minor adjustments: Analysis was conducted in-well and primary antibodies were incubated for two nights to permit thorough staining.

Quantification of antibody expression

The expression of the antibodies was quantified using the Axiovision program. Briefly, the area of the antibody was determined for each image (the Cy5 channel) and this was divided by the total area of the cells (the FITC channel). At least 5 images were quantified per neurosphere and at least 5 neurospheres on each coverslip were quantified. Coverslips were in triplicate for each antibody, and the experiment was repeated thee times. The data was then reported relative to the lowest expression with a grading system. (+++) represents at least three times the expression of the lowest expressing group. (++) represents at least two times the expression of the lowest expressing group, (+) represents positive expression.

Migration analysis

5× magnification images of entire neurosphere and surrounding cells that migrated from sphere were obtained. Migration of cells was analyzed using Axiovision software measurement tool. For each neurosphere, two measurements were obtained: the radius of the neurosphere and then the radius from the center of the neurosphere to the farthest point of the most distant cell. The difference between these radii was expressed as the distance of migration. This approach was chosen in order to control for the angle of the line that measured the extent of migration. The edge of the neurosphere was based on the last confluent layer of cells. The measurement was averaged for multiple neurospheres in each experimental group.

Neurite extension analysis

Neurofilament stained cells from 2D co-culture experiments were imaged and analyzed using MetaMorph (MDS Analytical Technologies) computer program, courtesy of Charles Greer, for average neurite length per cell in 20× frame. 18-bit grayscale images were analyzed according to preset parameters defining minima and maxima for cell body size, nuclear size and neurite outgrowth widths and lengths. Settings were also defined for positive staining based on threshold intensity level above background. The following parameters were used: Cell bodies: Max width = 40μm; Intensity above background = 50 gray levels; Minimum area = 20 μm^2. Nuclear Stain: Min width = 8μm; Max width = 30μm; Intensity above background = 33 gray levels. Outgrowths: Max width = 15μm; Intensity above background = 9 gray levels; Min cell growth as significant = 0μm. Experimental groups were compared with regards to total neurite outgrowth (average neurite length multiplied by average total number of neurites per cell.). This study was performed on images from both 3 day and 7 day experiments.

5-Bromo2′-deoxy-uridine (BrdU) proliferation assay

To quantify the proliferation of NSCs, a bromodeoxyuridine (BrdU) assay was used. After incubating NSCs in OEC CM or OEC Fractioned CM for 3 or 7 days, media from cells was removed and discarded. Cells were incubated in 100μM BrdU solution (Invitrogen) for 1.5 hours at 37°C. Cells were subsequently fixed in 10% Formalin then washed with 0.1M PBS and 1% Triton X-100 solution. Cells were incubated in 1N HCl for 10 minutes on ice, then with 2N HCl for 10 minutes at room temperature and 20 minutes at 37°C. HCl was removed and borate buffer (0.1M) added for 12 minutes at room temperature. Cells were washed and blocked with blocking buffer (0.1M PBS, 1% Triton X-100, 1M glycine, 5% NGS) for 1 hour at room temperature and incubated with anti-BrdU antibody (rabbit α BrdU, 1:1000) overnight. Antibody was detected with Alexafluor 647 antibody and visualized using Zeiss Axiovert microscope using Cy5 channel. The area of BrdU positive cells was measured per 20× frame and normalized by the area of GFP positive cells. Area fluorescence measurements were made using calibrated measurement software tool in Axiovision program. Area was used rather than number of cells as it was difficult to count cells in the neurosphere.

Statistical analysis

All experiments were performed in duplicate. One-way ANOVA statistical tests were used to analyze quantitative migration, proliferation and neurite extension data and differences between groups were compared using Bonferroni Test. Differences were considered significant if $P < 0.05$.

Results

OECs Promote the Differentiation of NSCs in a Three Dimensional Coculture System

In this work, we have used an olfactory ensheathing cell line. Isolation of OECs has the propensity for leading to multiple cell types [43–46], and the age and passage number of olfactory ensheathing cells can play a significant role in their behavior and protein expression [47–49]. Therefore, a number of groups have developed cell lines that exhibit consistent protein expression and behavior of their primary counterparts without the potentially confounding introduction of contaminating cells [43,50–53]. Since we wanted to investigate their impact on NSCs, it was critical to have one, specific OEC cell type. Therefore, we chose a line that has been shown to be pure and to have phenotypes identical to primary cell lines in a number of models [54–56].

To qualitatively evaluate the effect of a co-culture between NSCs and OECs, both cells were combined in growth factor reduced MATRIGEL ® matrix in the presence of serum-free media. At 11 days post seeding, NSCs grown alone in MATRIGEL remain small and circular (Figure 1A), but NSCs cocultured in the presence of OECs extend long neurites and exhibit typical glial and neuronal morphology (Figure 1B, C). At 14 days post seeding, the morphology of the NSCs cocultured with the OECs is even more striking (Figure 1D). Differentiation analysis was conducted using immunocytochemistry at 17 days post seeded. NSCs cocultured with OECs positively express glial fibrillary acidic protein (GFAP) (Figure 1E), neurofilament (NF) (Figure 1F), and beta-III-tubulin (B3T) and do not express nestin.

NSCs cultured in matrix alone weakly express B3T and GFAP and do not express NF. They do, however, positively express nestin. These results are summarized in Figure 1G.

These findings motivated our interest in exploring what role the OECs may be playing in directing such striking changes in morphology and differentiation of the NSCs.

OEC Conditioned Media Inhibits NSC proliferation

Proliferation of NSCs in OEC CM was characterized using BrdU analysis. In the OEC CM, NSCs had significantly fewer BrdU positive cells as compared to NSCs in OEC media and NSC media at both 3 and 7 day time points (Figure 2A). Significantly decreased proliferation in the OEC CM group suggests that NSCs are committing themselves to a glial or neuronal lineage. However, there were no significant differences between the OEC media and OEC CM media, which suggests that the inhibition of proliferation is not necessarily due to soluble factors.

OEC Conditioned Media Promotes the Differentiation of NSCs

To look at the commitment of NSCs to glial or neuronal lineages, we investigated the expression of several markers for differentiation. NSCs were cultured on poly-D-lysine/ laminin coated glass coverslips in the presence of OEC conditioned media, OEC media (control) and NSC media (control) for 3 and 7 days. In both the OEC conditioned media and OEC media (control) group, NSCs migrated extensively from the sphere and showed signs of differentiation as early as the 3 day time-point. Qualitative analysis of differentiation at the 7 day time point is summarized in Figure 2L.

Down regulation of nestin and up regulation of GFAP, NF and B3T in both OEC conditioned media and OEC media control groups was observed (Figure 2C-K demonstrate GFAP, NF and nestin staining patterns). Positive staining for GFAP, B3T and NF appeared more extensive in the conditioned media group than the OEC media control group. The NSC media group showed strong expression of nestin and weak expression of GFAP and NF. Each group also exhibited different morphologies. Cells in the OEC conditioned media group tended to have small cell bodies with numerous, long processes. Cells in the OEC media group had large cell bodies with fewer processes. Cells in the NSC Media group were small, round and usually lacking in neurites, even those that migrated from the center of the neurosphere.

These trends did not appear to change at the 7 day point. The proportion of GFAP and NF/B3T expressing cells in each group stayed roughly the same. We did notice that some of the processes in the conditioned media group were longer and some of the cells appeared closer to the phenotype of a mature neuron.

OEC Conditioned Media Promotes Neurite Extension

Cells in all groups at both the 3 and 7 day time points extended processes, though these varied tremendously in number and length. MetaMorph software analysis of NF-stained images quantified neurite length using shape-dependent extension recognition. Average neurite length differed significantly between all three groups at both the 3 and 7 day time

points. Interestingly, average total neurite growth nearly doubled in each experimental group between 3 and 7 days.

Migration of NSCs in Fractioned OEC Conditioned Media after 7 Days

To investigate the relative sizes of factors secreted by OECs which are responsible for increasing the migration of NSCs, cells were incubated in fractioned OEC CM. Four fractions were generated using centrifugation filter tubes: 0–10kDa, 10–30kDa, 30–100kDa and >100kDa. After seven days, cells were visualized with a fluorescent microscope and migration of cells from the sphere was measured. NSCs migrated on average 265μm in the 0–10kDa fraction, 275μm in the 10–30kDa fraction, 375μm in the 30–100kDa fraction and 345μm in the 100+ kDa fraction (Figure 4D). NSCs migrated the furthest in the 30–100kDa group. However there was no significant difference in migration of cells between groups.

Proliferation of NSCs in Fractioned OEC Conditioned Media after 7 Days

Proliferation of NSCs in fractioned OEC CM was characterized using BrdU analysis. After seven days, cells were incubated with a labeled thymidine analog to mark actively dividing cells. BrdU positive cells were detected using fluorescent microscopy. Significantly more control NSCs incubated in NSC media were BrdU positive compared to all fractioned OEC CM groups, unfractioned CM group, and OEC media control groups (Figure 3). NSCs in >100kDa fractioned OEC CM exhibited very minimal proliferation after seven days, the least of all fractioned OEC CM groups.

Differentiation of NSCs in Fractioned OEC Conditioned Media after 7 Days

Differentiation of NSCs in fractioned OEC CM after 7 days was characterized using immunocytochemistry. NSCs incubated in four different fractioned OEC CM groups, as outlined previously, were stained for nestin, NF, GFAP, and B3T. Using these antibodies, we were able to characterize the extent of differentiation of NSCs in fractioned OEC CM. Progressively increasing NF expression was observed in fractions with increased molecular weight cutoffs. Very little NF expression was observed in the 0–10kDa fraction. Increased nestin expression was observed in the 0–10kDa fraction and limited expression equally in 10–30kDa, 30–100kDa and >100kDa fractions. GFAP expression was low in the 0–10kDa fraction, and strongly expressed in the remaining fractions (Figure 3). B3T expression was not observed in the 0–10kDa and 10–30kDa fractions, minimally observed in the >100kDa fraction, and consistently observed in the 30–100kDa fraction (Figure 3). Morphologically, NSCs in 0–10kDa fractioned OEC CM were small and extended few neurites. NSCs in fractioned OEC CM containing proteins greater than 30kDa extended long and multiple neurites with large growth cones. Together, these results indicate that large proteins greater than 30kDa are contributing to the differentiation of NSCs in as little as 7 days. Qualitative observations are summarized in Table 3.

OEC Conditioned Media Promotes the Migration of NSCs

The migration of NSCs was analyzed after 3 and 7 days. Because we utilized NSCs expressing GFP, we were able to visualize and photograph the neurospheres using a fluorescence microscope equipped with a FITC filter. To measure the extent of a migration,

we first measured the radius of the neurosphere and subsequently the distance from the edge of the sphere to the furthest neurite (Figure 4A). After three days, NSCs in OEC CM migrated significantly greater distances than NSCs in either NSC media or OEC media alone (Figure 4B). NSCs incubated with OEC CM migrated an average distance of 375μm. Control NSCs incubated in NSC media or OEC media alone migrated on average distance of 150μm and 200μm respectively. After seven days, NSCs in OEC CM migrated significantly farther than NSCs in either control group (Figure 4C). NSCs incubated with OEC CM migrated an average distance of 500μm. Control NSCs incubated in NSC media or OEC media alone migrated on average distance of 75μm and 100μm respectively. Likewise, we saw the greatest migration in the high molecular weight cutoff groups of OEC CM (figure 4D).

OEC Conditioned Media Promotes Synapsin-1 Expression in NSCs after 7 Days

We used immunocytochemistry to test for expression of a panel of factors including neurotransmitters and factors regulating neurotransmitter synthesis and release: GABA, tyrosine hydroxylase, choline acetyltransferase, GAD 65/67, glutamate, Synapsin I. We stained cells at the 3 and 7 day time point. Positive staining was detected for Synapsin I at the 7 day time-point (Figure 5) in NSCs treated with OEC CM. Synapsin I, a protein that regulates neurotransmitter release, was found in the processes of several cells with neuronal morphologies. It was not detected in either the OEC media or NSC media groups. We did not detect the other neurotransmitters--which is not surprising since they develop later in neurogenesis.

Discussion

To study the effect of a co-culture between NSCs and OECs, we first investigated the extent to which NSCs differentiate in the presence of OECs in a three dimensional matrix. Using immunocytochemistry, we found that OECs promote the differentiation of NSCs as marked by increased expression of NF, GFAP and B3T and decreased expression of nestin. We also observed that OECs promote a significant change in the morphology of NSCs which resemble mature neurons and astrocytes. The extent of these morphological changes and extent of expression over such a short timer period are unique in our experience. We wanted to understand what it is about the coculture that drove such rapid and significant changes in the NSC morphology and marker expression.

To determine whether these effects are mediated by direct or indirect contact between NSCs and OECs, we utilized OEC conditioned media and demonstrate that soluble factors larger than 30kDa in size are released by OECs and promote the differentiation, migration, neurite extension, and maturation of NSCs in seven days.

Immunocytochemical analysis of NSCs incubated in OEC conditioned media suggests NSC development along both neuronal and astrocytic lineages, as evidenced by an up regulation of NF, B3T and GFAP and a down regulation of nestin. These results are supported by the differences detected in neurite extension, migration and proliferation, which were significant between the experimental and control groups and across time points. In addition, the detection of synapsin-1, a protein associated with neurotransmitter release, demonstrates

advanced differentiation in the neuronal lineage for NSCs exposed to OEC conditioned media. The timeline of these results was particularly striking, since NF positive cells began to appear as early as the 3 day time point, evidence of neurotransmitter release was found as early as 7 days and neurite extension doubled from the 3 to 7 day time point. Differentiation appears to occur at a rapid rate. Importantly, in our immunocytochemistry, migration, proliferation and neurite extension trials, NSCs incubated in unconditioned OEC media or NSC media show a decreased level of differentiation, migration, and neurite extension and show an increased level of proliferation as compared to the OEC conditioned media group. These differences were statistically significant and are bolstered by the visible morphological differences observed in immunocytochemistry. Taken together, the evidence suggests a crucial change in the developmental path taken by NSCs exposed to OEC soluble factors and those that were not. These controls rule out the potential interference of other experimental factors known to promote differentiation, namely the laminin coated on the coverslips and the serum in the OEC media.

With these results, we are the first to establish the positive effect of OECs on the differentiation of NSCs *in vitro* through indirect contact. Several groups have previously speculated on the potential synergistic effects of their combination [57,58] but none have shown direct or indirect positive influence on differentiation. The response of NSCs to OEC conditioned media in this study disagrees with the findings of a recent *in vitro* study suggesting that OECs have an inhibitory effect on neuronal differentiation and instead promote NSC proliferation [59]. Zhang et al. [59] also found that the majority of NSCs in the NSC media control group differentiated into GFAP positive cells at the 8 day time point, in contrast to the limited differentiation of any kind observed in the NSC media group of our study. However, we believe that these discrepancies result from different approaches. The study by Zhang et al. [59] used a different experimental setup and method of analysis, particularly cell-counting methods to determine expression that are frequently given to bias. They also focused on the effect of OECs on endogenous spinal cord NSCs. These results are intriguing because previous studies have determined that OECs exert their trophic effects through the release of smaller growth factors such as nerve growth factor (NGF) (26.7 kDa) and brain derived neurotrophic factor (BDNF) (27 kDa) [48]. OECs have been previously shown to support the migration of Schwann cells by secreting NGF [60]. Smaller proteins implicated in supporting neurogenesis include cilliary neurotrophic factor (CNTF) (22.9 kDa), neurturin (NTN) and glial cell derived neurotrophic factor (GDNF) - all of which have been shown to be secreted by OECs [48]. Our studies, however, indicate that larger proteins may also be secreted by OECs.

OECs are known to secrete several large soluble factors that may play critical roles in driving the neural response seen here. These molecules include SPARC, Sonic hedgehog protein (Shh), matrix metalloproteinase 2 (MMP 2), fibronectin, and laminin. SPARC has a molecular weight of 43 kDa and has been shown to be secreted by OECs and implicated in neural differentiation as well as neurite extension [61,62]. Shh with a molecular weight of 48 kDa is a potential candidate given its ability to induce differentiation of neural progenitor cells into neurons [63]. Shh has been well characterized and implicated in early neural

development. MMP 2 with a molecular weight of 67 kDa has been shown to be critical for neural cell migration and subsequent differentation in a number of tissues in vivo [64–66].

On the high end, the laminins range from 100 to 400 kDa and have been shown to be critical for neural progenitor migration [67,68]. Laminin is also critical for promoting neural progenitor differentiation [12]. Fibronectin with a molecular weight of 440 kDa has also been shown to promote neural progenitor migration [67–69], although its role in neural differentiation is not documented in the manner than laminin has been. Nonetheless, it may play a role.

It is likely that it is some combination of these factors that leads to the robust responses we have seen in this work and it very well may be that the amounts and presentation over time will be critical in mimicking this response in a model system. The differentiation and neurite extension of neural stem and progenitor cells has been studied for many years now, and no one factor or factors has been shown to have the response seen with coculture system. In looking at the higher molecular weight factors, we have not ruled out the possibility that smaller proteins may dimerize and elicit the observed effects.

Our results strongly suggest the potential of a co-transplantation of both OECs and NSCs in spinal cord injury or other neural injury environment. Both cell types have been shown to individually promote recovery in spinal cord implants. When combined in a co-culture implant, they may continue to work individually and collaboratively, providing an ideal environment for the repair of neural tissue. In particular, our studies illustrate the dramatic effect OECs produce upon the release of a complex mixture of soluble factors. Previous studies have shown that isolated growth factors such as CNTF can promote the differentiation of NSCs [70–74]. Additional factors, including GDNF, have been shown to promote axonal growth in spinal cord injury models upon local delivery [75]. The combination of growth factor, including BDNF and insulin growth factor-I (IGF-1), has also been shown to promote neural stem cell differentiation [76]. However, OECs may prove to be the ideal source of multiple soluble factors, including growth factors and potentially larger proteins such as Shh, that can promote NSC differentiation.

Although we have established NSC differentiation in response to OEC soluble factors, we need a more exact understanding of which percentage of cells commit themselves to which lineage. Because these experiments are meant to serve as a foundation for the development of a spinal cord implant, it will be important to know what proportion of NSCs become astrocytes and neurons when their environment is influenced by the presence of OECs. This information will also be interesting to compare to conflicting *in vivo* reports that NSCs implanted on their own commit to either mainly astrocytes [32,33] or neurons [30,31].

Future experiments should also investigate the interaction of both OECs and NSCs with various biopolymers. Previous studies have experimented with *in vitro* and *in vivo* models of OECs seeded on collagen scaffolds [77] and matrigel [78] and NSCs seeded on electrospun nanofibers [79] and hydrogel [80]. With these experiments, an optimal implant for spinal cord injury can be designed.

Conclusions

Overall, we have shown that coculture of OECs and NSCs leads to robust differentiation of NSCs along with neurite extension that is truly unusual for NSCs in vitro. While we have yet to identify the specific molecules, we know that molecules greater than 30 kDa play the greatest role in these processes. This work provides the basis for pursuing two major therapeutic paths: engineering three dimensional coculture systems to promote repair following CNS injury, and the identification and application of large molecules to promote repair via endogenous cells in the CNS.

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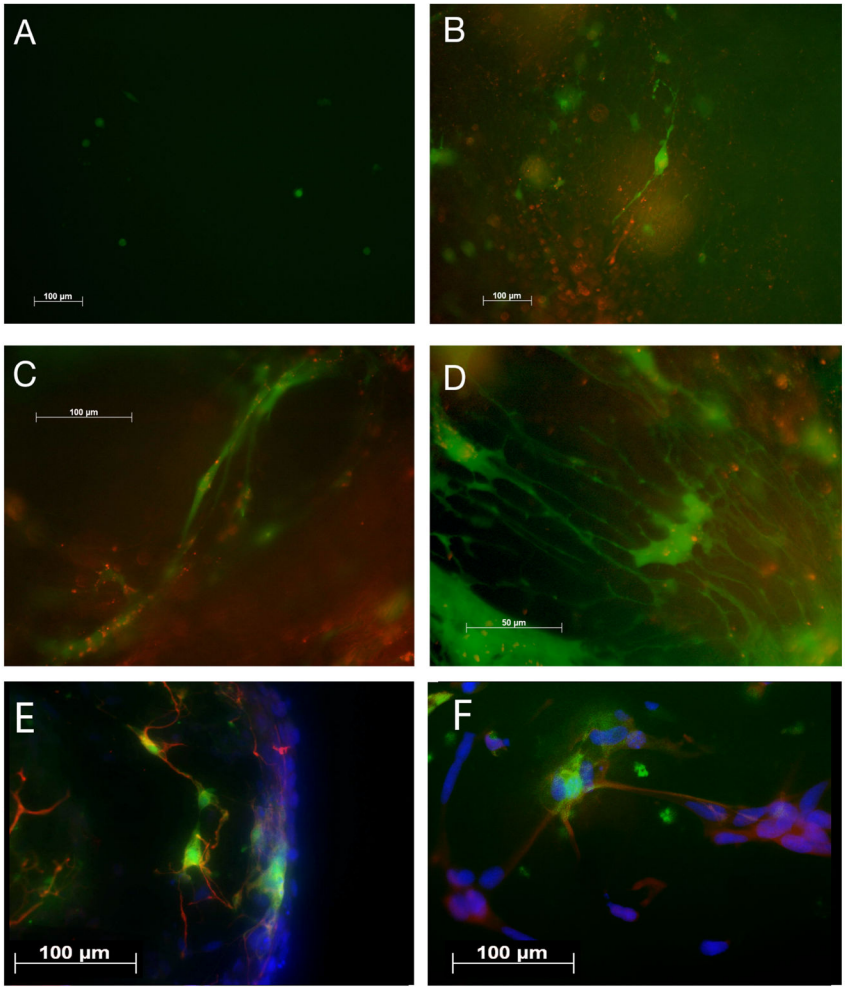
Summary

- Coculture of OECs and NSCs in three dimensional gels leads to robust neurite extension from the NSCs and neural differentiation
- The addition of conditioned media from OECs can recapitate this effect in 2D with NSCs
- This suggests that paracrine signaling is critical
- The addition of fractioned media from OECs allowed us to determine that the largest fraction (greater than 30 kDa) is critical for the NSC behavior
- This is larger than most growth factors currently identified as key for such behavior
- This suggests that looking at larger molecules such as sonic hedgehog could be key for developing new interventions
- Furthermore, coculture of OECs and NSCs could lead to powerful new therapies for CNS injuries



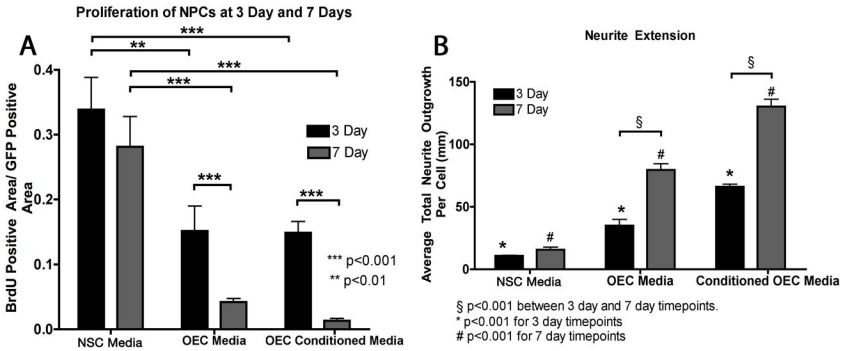
G

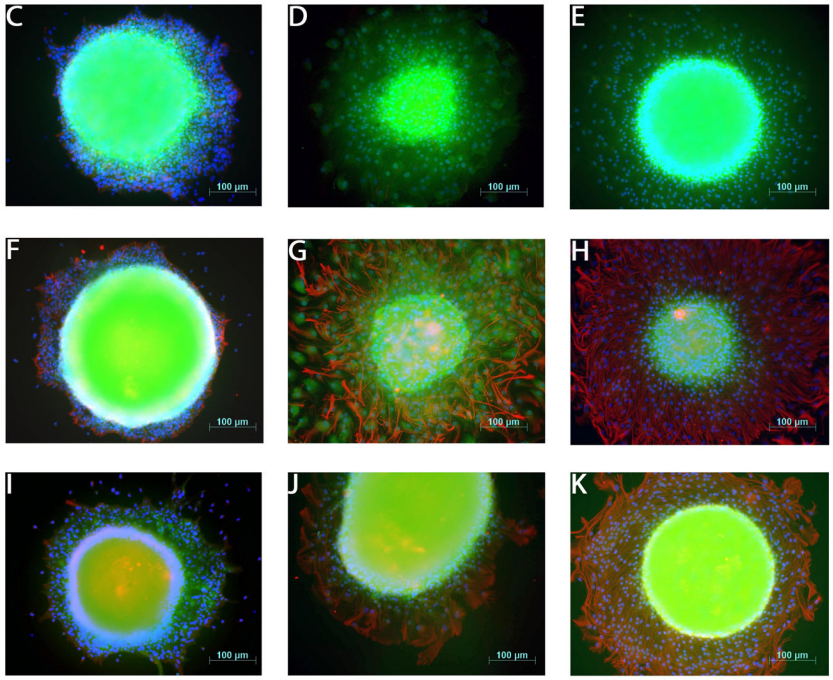
	NSCs Alone	NSCs (w/ OECs)
NF	--	+++
Nestin	+++	--
B3T	+	+++
GFAP	+	+++

Figure 1.

NSC morphology in Matrigel in the presence of OECs. (A) NSCs alone in matrigel, 11 days post seeding. The NSCs are alive and viable but showing limited interaction in the Matrigel and exist primarily as single cells with no neurites. (NSCs are GFP positive and green.) (B) 1:1 coculture of NSCs and OECs at 11 days post seeding. (NSCs are GFP positive and OECs have been incubated with DiI (red).) (C) Another example of the 1:1 coculture of NSCs and OECs at 11 days post seeding. (NSCs are GFP positive and OECs have been incubated with DiI (red).) (D) 1:1 coculture at 14 days post seeding. In the presence of OECs in the Matrigel, the NSCs extend many processes and appear to interact with each other. (NSCs are GFP positive and OECs have been incubated with DiI (red).) (E) NSCs at 17 days post seeding in Matrigel express GFAP (red) in the presence of OECs (1:1

coculture. (F) NSCs cocultured with OECs exhibit immunostaining for NF (red). (G) Expression of NF, nestin, beta$_{III}$ tubulin, and GFAP in NSCs alone in matrigel versus NSCs cocultured with OECs in matrigel at 17 days post seeding. The presence of OECs leads to a significant increase in markers for differentiation of the NSCs.



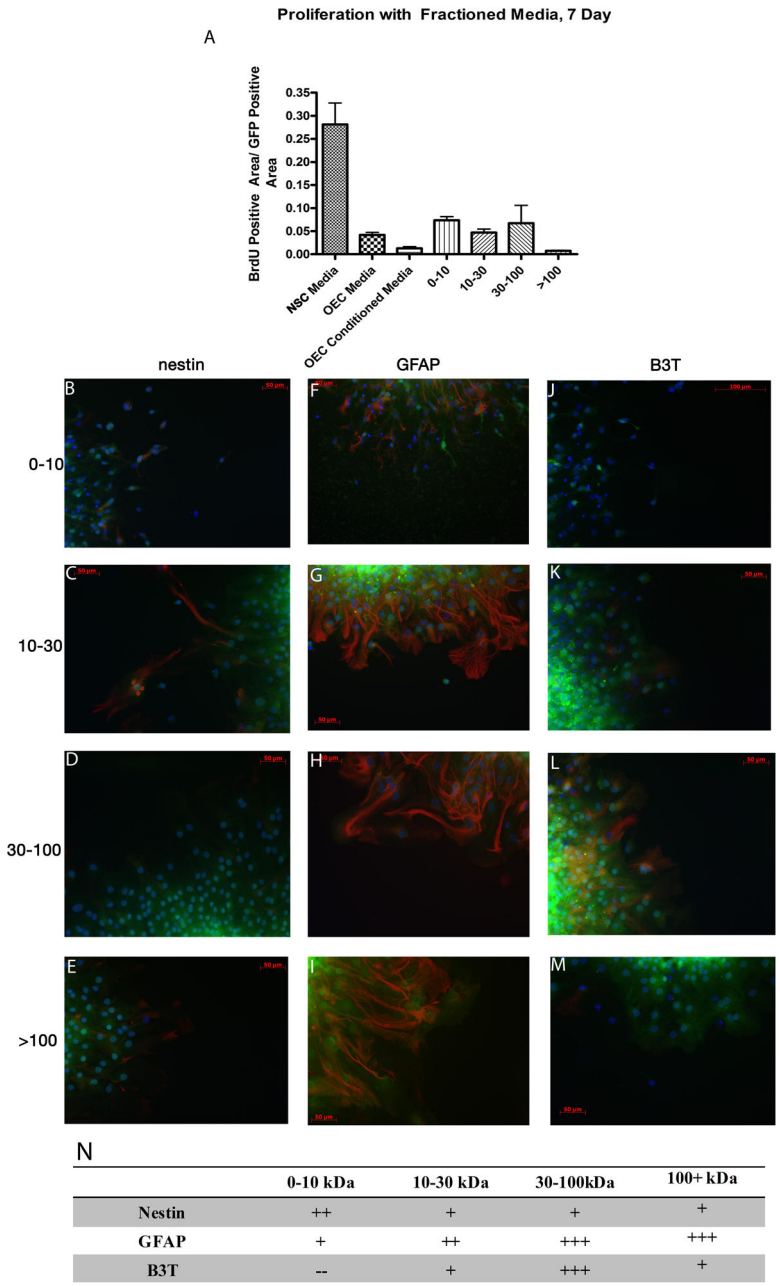


L Expression of Markers for Differentiation

	OEC Conditioned Media	OEC Media	NSC Media
Nestin	+	+	+++
GFAP	+++	++	++
NF	+++	++	+

Figure 2.

Proliferation, Neurite Extension and Differentiation of NSCs in OEC CM. (A) NSCs in OEC CM proliferate significantly less than NSCs in NSC or OEC media at both 3 and 7 day time points. (B) NSCs in OEC CM extend significantly longer neurites at both 3 and 7 day time points. (C) NSCs in NSC media strongly express Nestin. (red). (D and E) NSCs in OEC media express little Nestin and NSCs in OEC CM (E) express no Nestin. (F) NSCs in NSC media weakly express GFAP (red) as compared to (G) NSCs in OEC media and. (H) NSCs in OEC CM strongly express GFAP (red). (I) NSCs in NSC media weakly express NF (red). (J) NSCs in OEC media moderately express NF (red). (K) NSCs in OEC CM strongly express NF. Qualitative results of marker expression summarized in (L).



N

	0-10 kDa	10-30 kDa	30-100kDa	100+ kDa
Nestin	++	+	+	+
GFAP	+	++	+++	+++
B3T	--	+	+++	+

Figure 3.

Proliferation and Differentiation of NSCs in Fractioned OEC CM. (A) NSCs in OEC CM with soluble factors greater than 100kDa proliferate the least. (B) NSCs in OEC CM with soluble factors between 0–10kDa strongly express Nestin (red). (C) NSCs in OEC CM with soluble factors between 10–30 kDa moderately express Nestin. (D-E) NSCs in OEC CM with soluble factors greater than 100kDa weakly express Nestin. (F) NSCs in OEC CM with soluble factors between 0–10 kDa weakly express GFAP (red). (G) NSCs in OEC CM with soluble factors between 10–30 kDa moderately express GFAP. (H-I) NSCs in OEC CM with soluble factors between 30–100kDa (H) and greater than 100kDa (I) strongly express GFAP. (J) NSCs in OEC CM with soluble factors between 0–10kDa do not express B3T (red). (K)

NSCs in OEC CM with soluble factors between 10–30 kDa weakly express B3T (red). (L) NSCs in OEC CM with soluble factors between 30–100 kDa strongly express B3T. (M) NSCs in OEC CM with soluble factors greater than 100kDa weakly express B3T. Nuclei are stained blue. Qualitative observations summarized in (N).

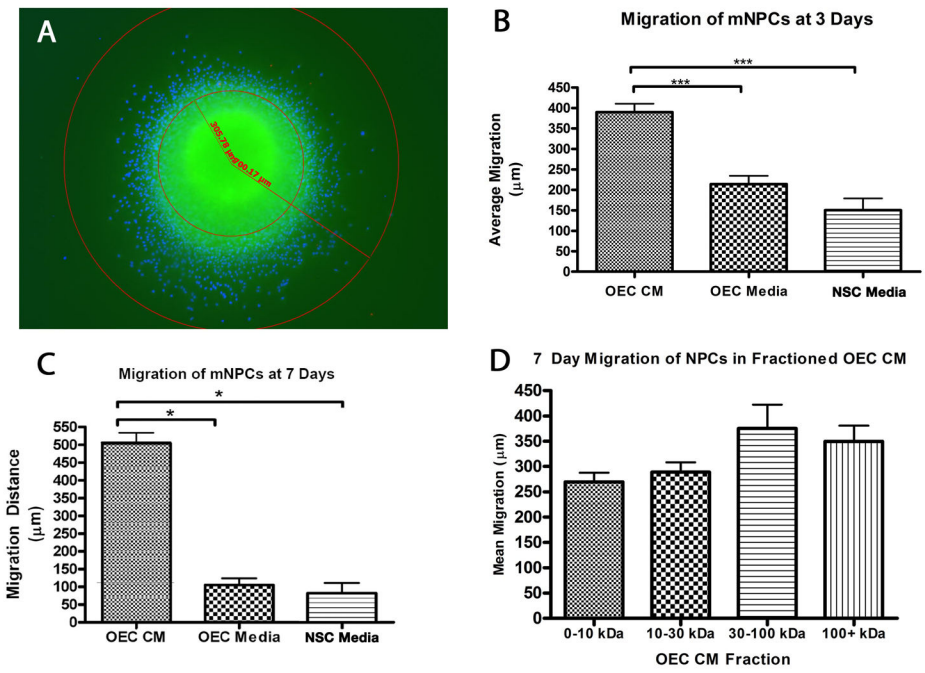


Figure 4.

Migration of NSCs. (A) Sample migration measurement illustrating technique using radius of neurosphere and radius of furthest neurite to compute migration distance. Scale bar = 200 um. (B) NSCs migrate significantly greater distances in OEC CM after 3 days as compared to controls. (C) NSCs migrate significantly more in OEC CM after 7 days as compared to controls. (D) Soluble factors between 30–100kDa released by OECs promote furthest NSC migration.

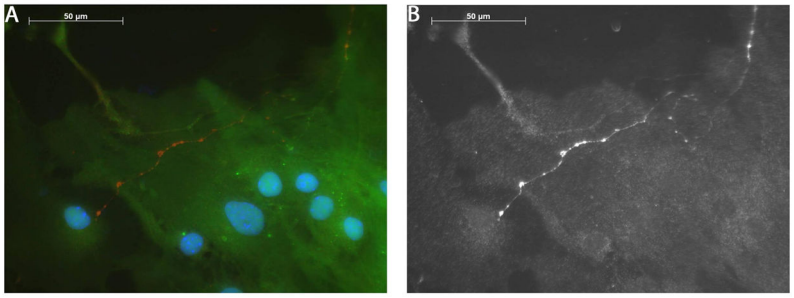


Figure 5.

Post-7 Day Synapsin-1 staining of NSCs treated with OEC CM. (A) 40× image of NSCs in OEC CM. Synapsin I expression (red) with characteristic vesicular staining along neurite. Nuclei are stained blue with DAPI and NSCs express GFP. (B) Grayscale image demonstrating positive staining.