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In vitro Maintenance of Olfactory Mucosa with Enriched Olfactory Ensheathing Cells

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Abstract

Human Olfactory Mucosa (OM) regulates olfaction through axonal regeneration and myelination mediated by stem cells and Olfactory Ensheathing Cells (OECs) resident in the niche. Purified OECs/olfactory biopsies have been utilized for functional recovery in different Spinal Cord Injury (SCI) models. However, recent reports find this debatable where we propose primary culture of OM, basal cells of olfactory epithelium and olfactory ecto-mesenchymal stem cells. Our defined culture conditions improve the life span of OM with enrichment of OECs providing a strategy for employment for SCI/cochlear damage repair.

Briefly, OM post-collection, was non-enzymatically sliced, cultured for 6 weeks and cells characterized morphologically, immuno-cytochemically and western blotting. By day 21, ~70% GFAP and p75NTR stained, spindle shaped astrocyte-like and flattened sheet-like OECs displayed axonal remyelination. By day 30, caspase 3, 8, 9 (gene-product and activity), phospho-p53 negative; GFAP and p75NTR positive dense, overlapping mass of cells was found. This was accompanied with degenerative changes by 6 weeks through GFAP staining. Conversely, trypsination on day 21 resulted in >95% OECs with flattened morphology, GFAP and p75NTR positivity. The human derived OECs were compared with the 2-day SD rat Olfactory Bulb Cells cultured for 2 weeks in F12 media (GFAP and p75NTR positive). Hence, cultured olfactory mucosa displaying axonal regeneration with OECs in culture provides a vehicle for SCI/cochlear damage repair studies.

Keywords: Olfactory ensheathing cells; Neonate Rat Olfactory Bulb; Human Olfactory Mucosa; Axonal regeneration; GFAP; p75^{NTR}

Introduction

The nasal mucosa regulates olfaction and is unique in its locale, neuronal characteristic communicating directly with external environs, easy extractability with functional loss, regeneration etc. Consequently, this tissue is adult stem cells repertoire for cell therapy.

The human olfactory mucosa consists of a pseudo-stratified columnar epithelium placed on a highly cellular lamina propria. The olfactory epithelium has four cell types: Ciliated Bipolar Olfactory Receptor Neuron, Sustentacular, Microvillar and Basal Cells [1]. The lamina propria contains Axon Fascicles, Blood Vessels, Connective Tissue, Olfactory Ensheathing Cells (OECs) and Bowman's Glands [1]. Recently, highly proliferative Olfactory Ecto-Mesenchymal Stem Cells (OE-MSCs) similar to bone marrow MSCs have been identified in lamina propria with a tendency to differentiate into neural cells [2]. Hence, the permanent neurogenesis (regenerate olfactory receptor neurons) owes to bi-compartmental stem cells of the mucosa, namely the basal cells of olfactory epithelium and OE-MSCs in underlying lamina propria. The OECs-a unique line of glial cells support the transition of axons of the olfactory receptor neurons from the peripheral nervous system (olfactory epithelium) to the central nervous system (olfactory bulb) [1]. Principally, OECs share similar phenotypic properties with both Astrocytes and Schwan cells have become a potential candidate for the transplant-mediated repair of the damaged Spinal Cord Injury (SCI) [3-5]. Further, the autologous, migratory and mingling property of OECs in central nervous system may provide an appropriate niche for the development of neurons [6]. OECs can be identified immuno-cytochemically through Low-Affinity Nerve Growth Factor Receptor p75 (p75NTR) and Glial Fibrillary Acidic Protein (GFAP) [6]. Their plasticity has also been described through altered morphology and antigen expression in response to culture media composition [7,8] cell density [9] and axon contact [10]. More, OECs also phagocytize cellular debris after injury *in vivo* [11] or *in vitro* [12]. Taken together, the above observations imply that OECs are regulated by the environment created by its adjoining cell types as well as culture conditions. Therefore, mixed culture population of enriched OECs under specified culture conditions in the niche of different stem cell types (Basal cells, OE-MSCs) may prove a promising approach for cell therapy during spinal cord injury.

Hence, the manuscript describes the culture of explants of human

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OM through middle turbinate region in a defined culture media. The method was first established with two-day-old Sprague-Dawley Neonate Olfactory Bulb derived OECs. Briefly, after collection, the olfactory mucosa was sliced without any enzymatic treatment and cultured for 40 days. At day 21, the process of neurogenesis and axonal remyelination was observed morphologically. Besides >95% of purified population of OECs with flattened morphology/GFAP/p75^{NTR} positivity was observed on trypsinization. Hence, direct transplantation of cultured OM in spinal cord injured patients compared to purified OECs may be a better approach.

Materials and Methods

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12, N-[2-Hydroxyethyl] piperazine-N′-2-ethanesulfonic acid (HEPES), Penicillin G, Streptomycin sulfate, Gentamicin sulfate, Dulbecco's Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), Human Epidermal Growth Factor (EGF), Human Transforming Growth Factor-α (TGF-α), Cholera Toxin, Hydrocortisone, Non-Essential Amino Acids were purchased from Sigma Chemical Co., St. Louis, MO, USA. Fetal Calf Serum (FCS) was procured from GIBCO BRL Laboratories, New York, USA. Antibodies against Active Caspase-3, Active Caspase-8 and Active Caspase-9 were purchased from BioVision Research Products (Mountain View, CA, SA). FITC labelled GFAP and p75^{NTR} were procured from Abcam (Cambridge, MA 02139-1517, USA). Protease and Phosphatase inhibitor cocktails, Cell Lysis M™ Cell Lysis reagent, antibodies against β-actin, p53, phospho (Ser-15) p53, and ExtrAvidin® Alkaline Phosphatase Staining kit as well as BCIP/NBT tablets were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Surgical extraction and culture of olfactory bulb of 2 day-neonate

2-day Neonate Sprague–Dawley was procured from the Division of Laboratory Animals from our institute maintained under standard animal care conditions. The study was approved by the Institutional Animal Ethics Committee. The animals were gently wiped with 70% ethanol in a laminar flow for ensuring aseptic conditions and decapitated. The olfactory bulbs were harvested after rapid removal of the skin and skull. The bulbs excised from six neonates were placed in a chilled 35-mm petri dish containing 2 ml of Ham's F12 medium, pH7.4 penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (60 μg/ml) supplemented with 10% FCS and 10 mM HEPES. The tissue was finely macerated and transferred to a T-25 flask/6-well plate and incubated at 37°C in the humidified 5% CO_2. The OECs were morphologically characterized on day 5, 9, and 13 through Nikon Eclipse Ti Phase Contrast Microscope and photographed. In addition, the confluent cells were trypsinated and immunocytochemically characterized on day 13 for Glial Fibrillary Acidic Protein (GFAP) and Low-Affinity Nerve Growth Factor Receptor p75 (p75^{NTR}). The flasks were left undisturbed with adequate medium, routinely examined for any apparent signs of contamination for allowing them to adhere to the substratum. Having ensured the attachment of cells until day 5, the medium was replaced with fresh medium each day.

Collection and culture of human olfactory mucosa (OM)

Two square millimeters OM was obtained from the root of the medial aspect of the middle turbinate endoscopically through ethmoid forceps from 35 and 65 year males with informed consent. The tissue was then transferred into a sterile 2 ml tube filled with chilled F12 and transported on ice removing the blood clots and debris through repeated washings. The tissue was explanted into 1mm^3 and placed in T-25 culture dish in 1:1 Dulbecco's Modified Eagle's medium (DMEM)/Ham's F12 containing penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (60 μg/ml), 10 μg/ml Bovine Insulin, 20 ng/ml Human Epidermal Growth Factor (EGF), 20 ng/ml Transforming Growth Factor-α (TGF-α), 100 ng/ml Cholera Toxin, 500 ng/ml Hydrocortisone, 82 mg/ml Non-Essential Amino Acids (NEAAs), 10% FCS and 10 mM HEPES in a humidified 5% CO2 incubator at 37°C. The flask was left undisturbed until day 9 to allow adherence to the substratum in ample media and checked for any visible signs of contamination. The emerging cells (day 9) were examined and photographed on day 9, 21, 30 with immunostaining at the latter two time points. In addition, trypsination was conducted on day 21 when confluency was achieved. Similarly, trituration of the confluent flask was also attempted with the purpose of negating the stress of enzymatic process and comparing the results morphologically with immunostaining.

Immunocytochemical staining of olfactory ensheathing cells

The extent of Olfactory Ensheathing Cells (OECs) present in the cultured explants was specifically immunostained with Glial Fibrillary Acidic Protein (GFAP) and Low-Affinity Nerve Growth Factor Receptor p75 (p75^{NTR}) antibodies.

Day 13 and Day 21 cells in neonate rat OB and human OM were fixed with chilled 3.7 % formalin in PBS at room temp and added 1ml of chilled methanol (-20°C) for 1 minute each. The methanol was removed & replaced with chilled PBS to prevent drying. To block nonspecific binding, added ~10ml (=1%) of goat serum was added and further incubated for 30 minutes at room temperature. The samples were probed with the appropriate primary antibodies for 1 hour at room temperature with gentle shaking. All primary antibodies were used at recommended dilution in 1% Bovine Serum Albumin in PBS. Finally, the cells were stained with FITC conjugated secondary antibody for 1 hour at room temperature followed by subsequent washings with chilled PBS. The cells were examined and photographed by a Nikon Eclipse Ti Inverted Phase Contrast Microscope.

Western blotting

1.0×10^6 cells were cultured in T-25 flasks until day 30. Pellet was disrupted in lysis buffer acquired from Cell Lysis M ™ Cell Lysis reagent, Sigma Chemical Co. (St. Louis, MO, USA) with protease and phosphatase inhibitor cocktail as per manufacturer's protocol. The supernatant was estimated for protein content [13]. 30 μg of protein per lane was processed for western blotting by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with the appropriate primary antibodies against Active Caspase-3, Active Caspase-8, Active Caspase-9, p53, phospho (Ser-15) p53 and β-actin. All primary antibodies were diluted in 1% Bovine Serum Albumin in Tris buffered saline with 0.1% Tween™ 20. Reactions were visualized with biotin conjugated secondary antibody and subsequently with Avidin linked Alkaline-Phosphatase enzyme conjugate according to the manufacturer's protocol.

Results and Discussion

Of the two options available for obtaining OECs from the tissue, we chose the non-enzymatic method in which the tissue slices left undisturbed for five days causes adherence of the explants to the plastic surface and subsequent emergence of the cell population [14].

Our approach has also been reaffirmed by Delorme et al. [2] that non-enzymatic methods conserve the quality of the both stem cells and OECs. Explantation causes minimal trauma to the tissue which is critical for cell quality. However, procedural constraints and absence of cells emanating at day 0 did not permit immuno-cytochemical quantification of the component cell types.

Primarily we isolated, cultured and characterized the OECs from 2-day old Sprague Dawley Olfactory Bulb. The cells emanating from the undisturbed tissue mass were greater in magnitude than on the periphery as a function of growth by day 5 (Figure 1A). However, the rate of cell multiplication enhanced subsequently reaching > 75% and were confluent by day 9 (Figure 1B) and 13 (Figure 1C) respectively. Microscopic examination suggested interspersed, dense population of heterogeneous cells distinctly containing at least two cell types through spatial juxtapositioning of unstained and immunostained areas through GFAP (Figure 2A). Our results are consistent with those obtained by Pixley [3] and Huang et al. [15]; they have also suggested the presence of two major cell types. The smaller, spindle shaped astrocyte like cells and the flattened bigger structures resembling Schwann cells were also noticed in our studies (Figure 2B). Among this cell population, $p75^{NTR}$ specifically stained spindle-shape astrocyte like cells (Figure 2C). Interestingly, the dichotomy between two cell types; Schwann-OEC (spindle-like morphology), expressing both p75NTR [16] and GFAP [17] and astrocyte-like OEC (flattened morphology) expressing only GFAP [5] have also been explained by Barnett and Roskams [18] (Figures 2B and 2C) Conspicuously, the intensity of GFAP/$p75^{NTR}$ stain was relatively intense around the anchored, day 13 residual tissue mass inner-core cells than those in the immediate vicinity versus the periphery.

In the present study, in order to evaluate the yield of OECs, the complete human OM biopsy was cultured for 40 days. OM post-collection was non-enzymatically sliced cultured and characterized morphologically, immuno-cytochemically and through western blotting. Initially, we observed the spherical clusters (due to absence of enzymatic treatment) which were morphologically similar to those developed through olfactory ecto-mesenchymal stem cells (OE-MSCs) as reported by Delorme et al. [2] (Figure 3A). The human OM survived and proliferated optimally in culture medium giving rise to 20-30% cell population by day 9 when left undisturbed (Figure 3B). On day 21, the anchored tissue allowed copious emergence of densely juxtaposed cell population radiating all around (Figure 3C). In certain areas, a densely packed monolayer-like population with occasional presence of olfactory neuronal network is also noticeable in the same cell mass. In sparsely populated area, intense inter-cellular gap junctions containing cells resembling typical neuronal characteristics was noticed (Figure 3C). Parallel staining of day 21 samples revealed ~70% GFAP and $p75^{NTR}$ positively stained cell mass superimposable over unstained cells suggesting enrichment of OECs (Figures 4A and 4B). The cloistered manner in which majority of the cells assimilating GFAP and $p75^{NTR}$ is indicative of their wrap-around, ensheathing, encapsulating characteristic around neuronal network (Figures 4A and 4B). Physiologically this may be relevant to the known, spatial, directional migration of these cells in the neuronal differentiation. Both spindle shaped astrocyte like and the flattened sheet like structures resembling Schwann cells were also noticed (Figure 5). The composition of cells in the culture vessel was of single flattened sheet like morphology (Figure 6). By day 30, the cellular morphology transformed into a more firm sheet like structure throughout the area viewed with neurogenesis (Figures 7A-7D) owing to the presence of other cell types (fibroblast-like cells, mesenchymal stem cells, connective cells, immune cells and pericytes [19-21] with enriched OECs. Absence of Active Caspases 3, 8, 9 and phospho-p53 further supported the facts (Figure 8). Hence, we

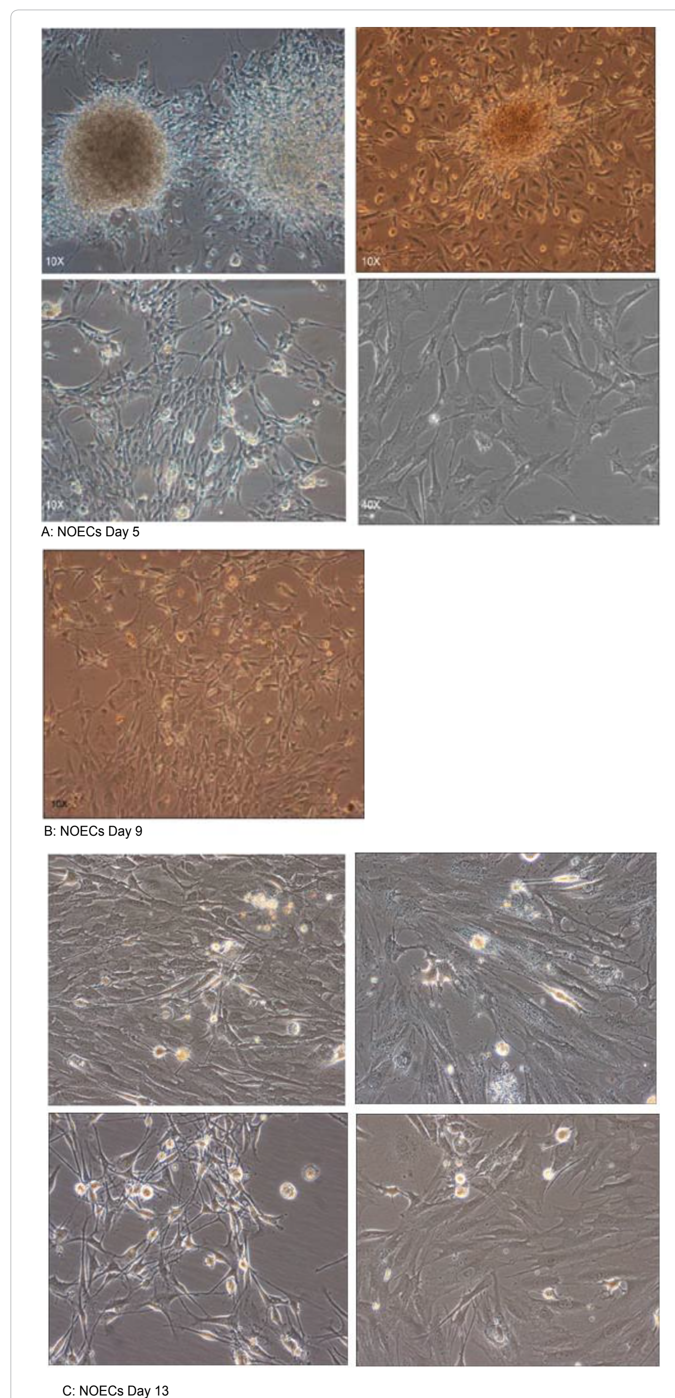


Figure 1: The finely macerated olfactory bulbs harvested from 2-day-old Neonate of Sprague–Dawley strain were placed in a T-25 flask containing F12 medium, pH7.4 at 37°C in the humidified 5% CO_2. The OECs, morphologically characterized on day 5 (A), day 9 (B) and day 13 (C; displaying confluency with presence of two major cell types, the smaller, spindle shaped astrocyte like cells and the flattened bigger structures resembling Schwann cells) through Nikon Eclipse Ti Phase Contrast Microscope and photographed with magnification 10X and 40X.

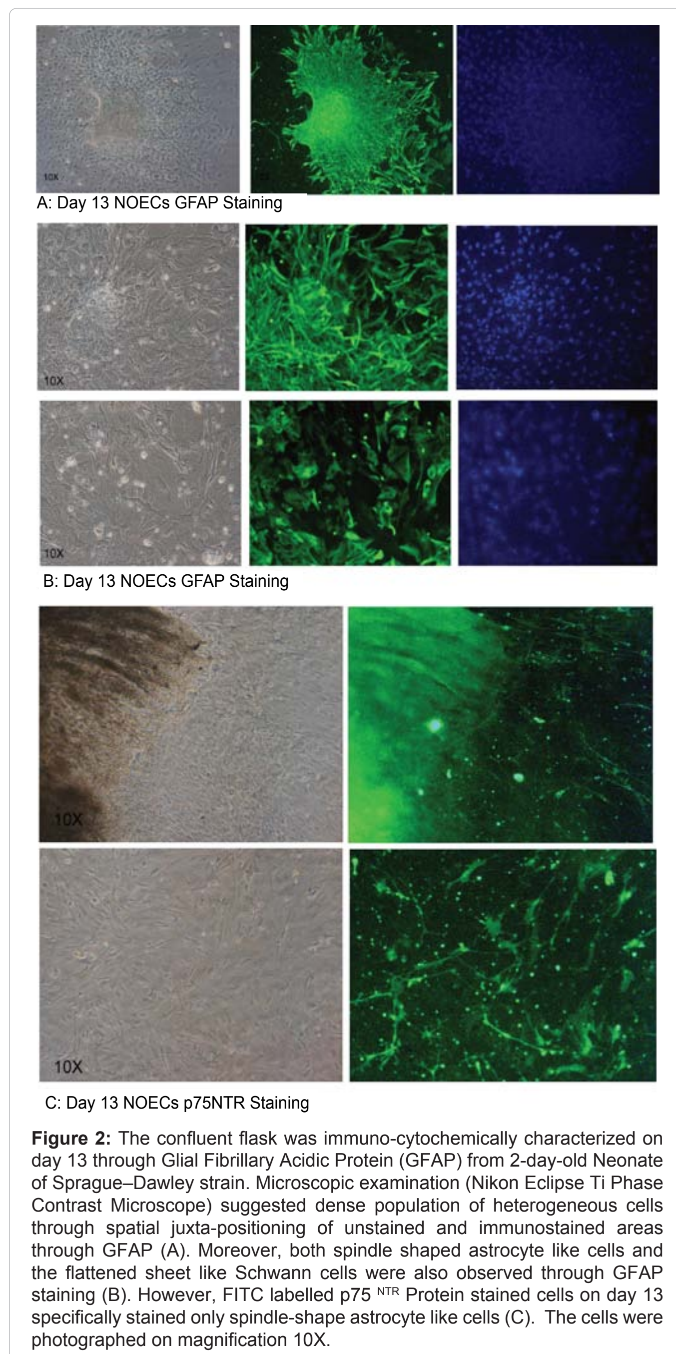


Figure 2: The confluent flask was immuno-cytochemically characterized on day 13 through Glial Fibrillary Acidic Protein (GFAP) from 2-day-old Neonate of Sprague–Dawley strain. Microscopic examination (Nikon Eclipse Ti Phase Contrast Microscope) suggested dense population of heterogeneous cells through spatial juxta-positioning of unstained and immunostained areas through GFAP (A). Moreover, both spindle shaped astrocyte like cells and the flattened sheet like Schwann cells were also observed through GFAP staining (B). However, FITC labelled p75 ^{NTR} Protein stained cells on day 13 specifically stained only spindle-shape astrocyte like cells (C). The cells were photographed on magnification 10X.

conclude that the defined media i.e. the supplemented growth factors in the primary culture allowed the cells to overcome the problem of limited life span and enriched the OECs. More, low concentrations of cholera toxin stimulated the cell growth and increased the number of passages in primary OM cells [22] through stimulation of basal and forskolin-amplified adenylate cyclase activity [23]. EGF a structural homologue of TGF-α, both exerting their actions through EGF receptor [24] were mitogenic to OM [25]. Insulin and hydrocortisone further supported the proliferation and differentiation of the cells [26]. However, these growth factors did not modulate the expression of p75^{NTR} and GFAP staining except that they stimulated the proliferation/stratification until 6 weeks and passaging on day 21 which stopped in serum alone on 30th day [5]. Further, by 6 weeks the cultured cell mass displayed

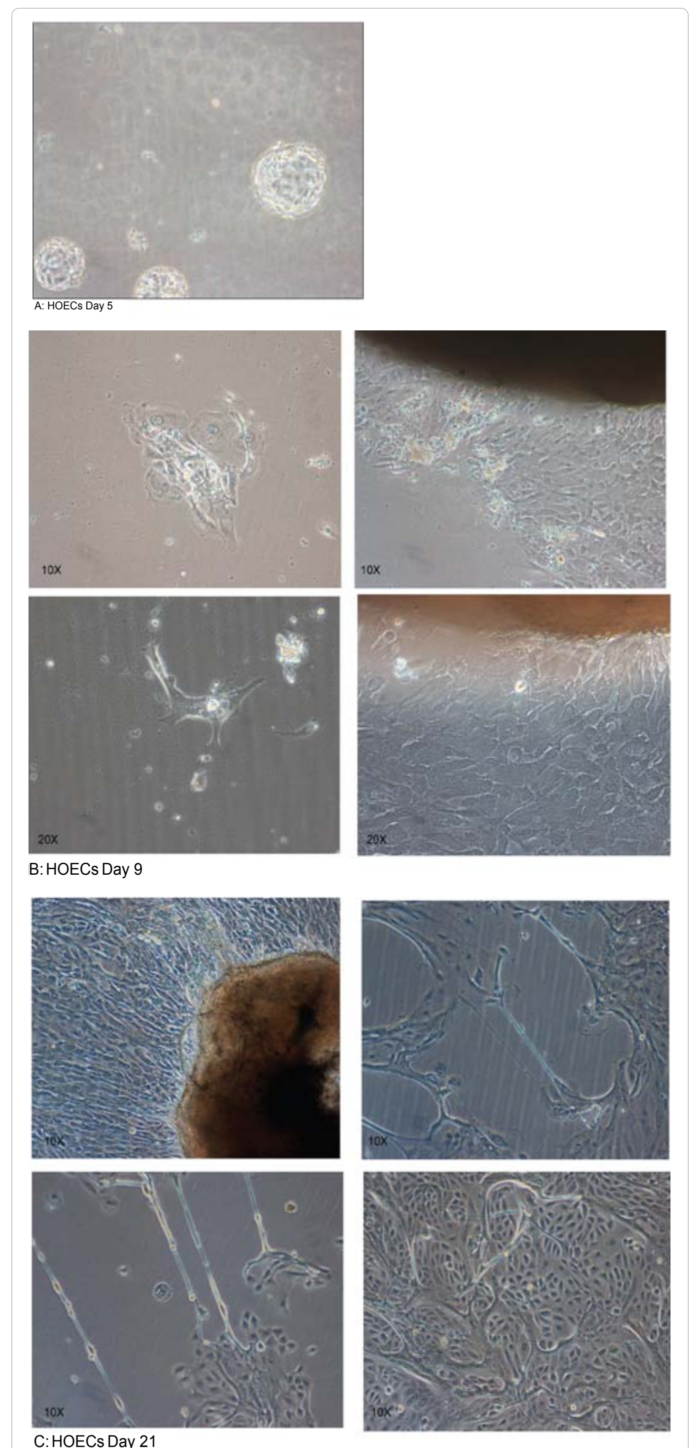
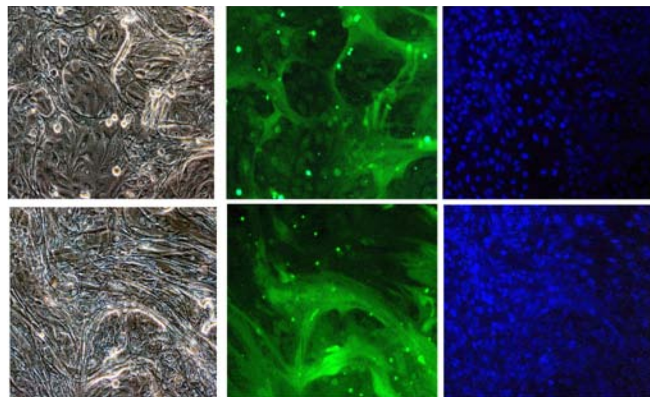
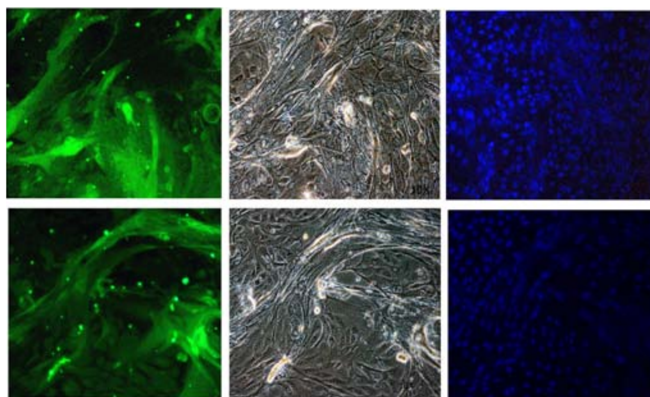


Figure 3: The human Olfactory mucosa (OM) explants were placed in T-25 flask in 1:1 Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 containing penicillin, streptomycin, gentamicin, bovine insulin, Human epidermal growth factor (EGF), Transforming Growth Factor-α (TGF-α), cholera toxin, hydrocortisone, Non-essential amino acids (NEAAs), 10% FCS and 10 mM HEPES in a humidified 5% CO_2 incubator at 37°C. Spherical clusters were observed on day 5 (A), a mixed population of cells emanating around the explants from the undisturbed flask on day 9 (B) and the attached explants allowed abundant emergence of dense cell population radiating all around with axonal junctions in sparse populated area with occasional presence of olfactory neuronal network is also marked on day 21 (C). The cells were photographed through Nikon Eclipse Ti Phase Contrast with magnification 10X.

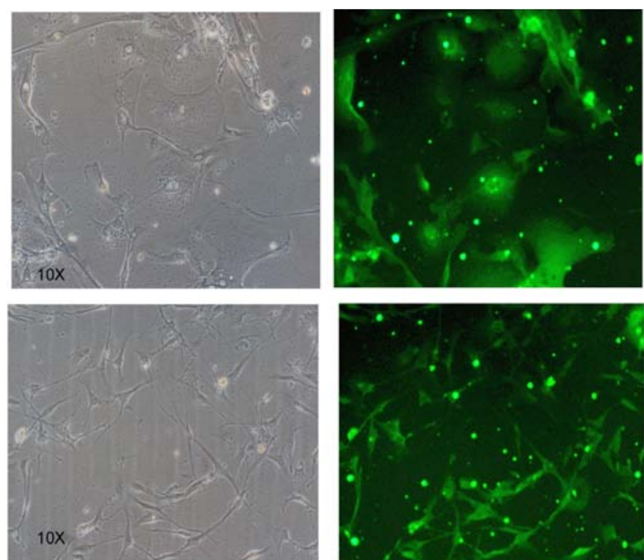


A: HOECs Day 21 GFAP Staining



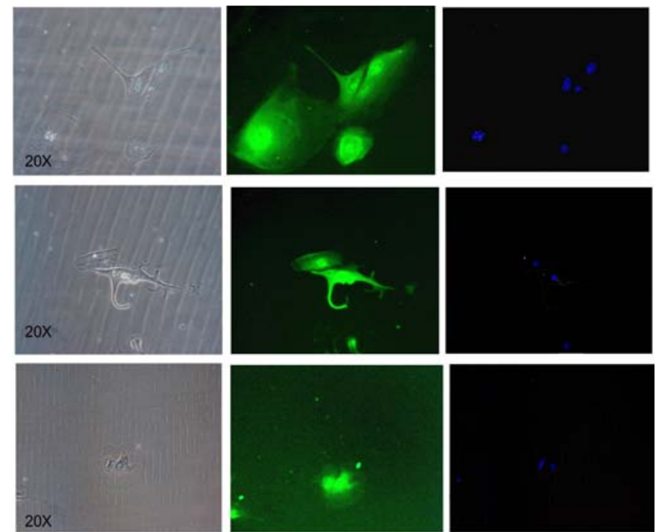
B: HOECs Day 21 p75NTR Staining

Figure 4: FITC labelled Glial Fibrillary Acidic Protein/p75^{NTR} staining of day 21 culture revealed ~70% GFAP (A) and p75^{NTR} (B) positively stained cell mass super imposable over unstained cells suggesting enrichment of OECs. The ensheathing characteristic around neuronal network is also evidenced. The photographs were acquired by Nikon Eclipse Ti Phase Contrast with magnification 10X.



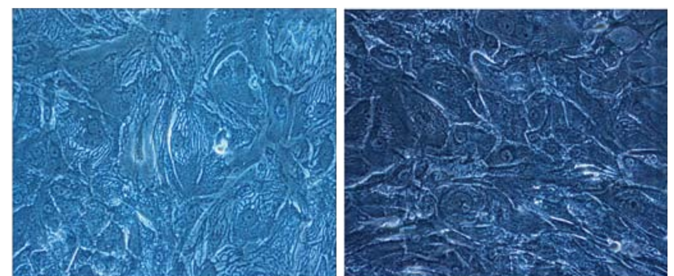
HOECs Day 21 p75NTR Staining

Figure 5: FITC labelled p75^{NTR} Protein staining sparse area of flask on day 21 showing both spindle shaped astrocyte like and the flattened sheet like structures resembling Schwann cells were also noticed. The photographs were acquired by Nikon Eclipse Ti Phase Contrast with magnification 10X.

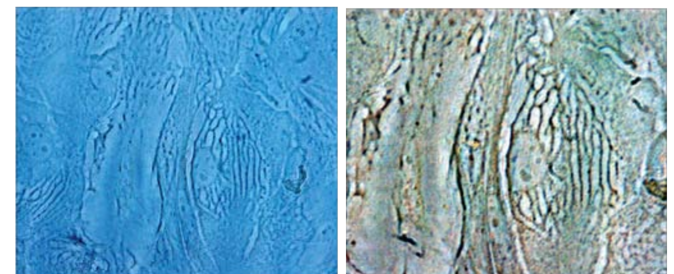


HOECs Day 21 GFAP Staining (single cell morphology)

Figure 6: The FITC labelled Glial Fibrillary Acidic Protein showing single flattened sheet like morphology as wells as depicting directional migration. The photographs were obtained by Nikon Eclipse Ti Phase Contrast with magnification 20X.



A: HOECs Day 30 (20X) B: HOECs Day 30 (20X)



C: HOECs Day 30 (40X) D: HOECs Day 30 (60X)

Figure 7: By day 30, the cellular morphology transformed into a more firm sheet like structure throughout the area viewed with neurogenesis. Axonal anastomosis was also observed with cell body validating the promotion of axonal growth and resultantly into complete olfactory neuro-epithelium (A (20X) and B (20X)). Enlarged view of cell body with axonal anastomosis (C (40X) and D (60X)). The photographs were acquired by Nikon Eclipse Ti Phase Contrast.

degenerative changes where the cell morphology appeared dissoluting proven through GFAP immunostaining and other supporting studies [12]. This is also in agreement with the phase contrast microscopy pictures (Figure 9).

Many reports have been published on culturing of OECs through OM biopsies since isolation of olfactory mucosa is a less invasive protocol. Most importantly, Choi et al. [27] reported differential yield

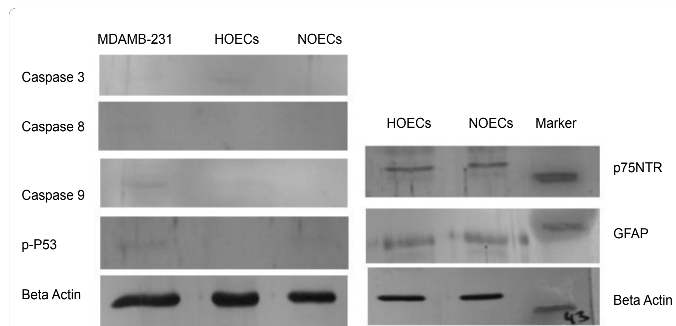


Figure 8: For expression analysis, cultured OECs (NOECs (day 13); HOECs (day 30) were cultured in in T-25 flask in 1:1 Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12 containing penicillin, streptomycin, gentamicin, bovine insulin, Human epidermal growth factor (hEGF), Transforming Growth Factor-α (TGF-α), cholera toxin, hydrocortisone, Non-essential amino acids (NEAAs), 10% FCS and 10 mM HEPES in a humidified 5% CO_2 incubator at 37°C. 30 µg of the whole cell lysate was separated on a 12.5% SDS-PAGE gel, probed with GFAP, $p75^{NTR}$, caspase 3, 8, 9 and phospho-p53 antibodies following transfer to a nitrocellulose membrane. β-actin was used as a protein loading control. Data shown is representative of one of the three similar experiments each performed in triplicate.

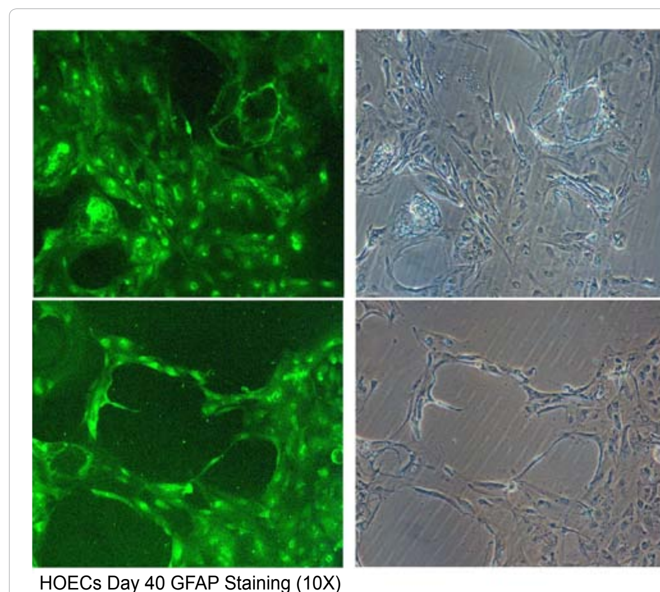


Figure 9: By 6 weeks the cultured cell mass demonstrated degenerative changes where the cell morphology appeared dissoluting established through FITC labelled GFAP. This is in agreement with the unstained phase contrast microscopy pictures observed at 10X.

of OECs from different regions of nasal septal mucosa. However, the major difference with us is that they enzymatically treated the biopsy with collagenase which might have disrupted the lamina propria [28]. Following this proposition we processed our biopsy specimen without any enzymatic treatment which enabled us to obtain complete olfactory epithelium with axonal regeneration [29]. Lima et al. [30] and Chabbra et al. [31] utilized acutely prepared minced OM tissue in Spinal Cord Injury site without culturing them. However, neither they validated nor they enriched the OECs in biopsy specimens. Hence, low percentage of OECs/absence of OECs may have provided safe OM autograft transplantation, without establishment of any physiological efficacy [30,31]. Feron et al. [29] cultured OECs for about 28 days and found >95% GFAP-positive and 76 to 88% $p75^{NTR}$ cells which supports our study. They conducted a phase I safety study using purified suspension of autologous OECs and reported no undesirable effects without any neurological improvement at 12 months post-transplantation. Through above and our studies, we conclude and propose that OECs with other cell types, rather than pure OECs may produce optimal transplant-mediated repair. Li et al. [32] also supported the enhanced efficacy of mixed culture (50:50 ratios of OECs with olfactory nerve fibroblast-like cells) compared to pure OECs. Our findings help define appropriate conditions for OM attachment, initiation of cells, extrusion, proliferation, differentiation and migration. Additionally, enriched populace of OECs may prove their potential as therapeutic for human spinal cord injury and cochlear damage. However, there are several important practical issues that will need to be solved before they reach general clinical application.

Passaging of confluent cells

The cultured explants from the neonate rat OB versus human OM displayed heterogeneity in terms of multiplication of cells resulted in achievement of cell confluency by day 13 (Figure 10) and 21(Figures 11A and 11B) respectively. Following trypsination through standard procedures, the cells were microscopically examined and immunologically evaluated on day 4 (rat OB) and day 7 (human OM) respectively in the two cases. We found the cells resembling those from the parent flask without any apparent alteration (Figures 10, 11A and 11B). Different methods like differential adhesion of OECs with respect to other cells [33]; with Thy1.1 complement lysis [34]; through immunopanning (anti-p75NTR) [35]; using magnetic nanoparticles; through FACS [4] have been utilized for purifying OECs. However, we tried purification of OECs through differential adhesion. We enriched/cultured the OECs for day 21 and on trypsinization we obtained >90% population positive for p75NTR as well as GFAP in the flask (Figures 11A and 11B).

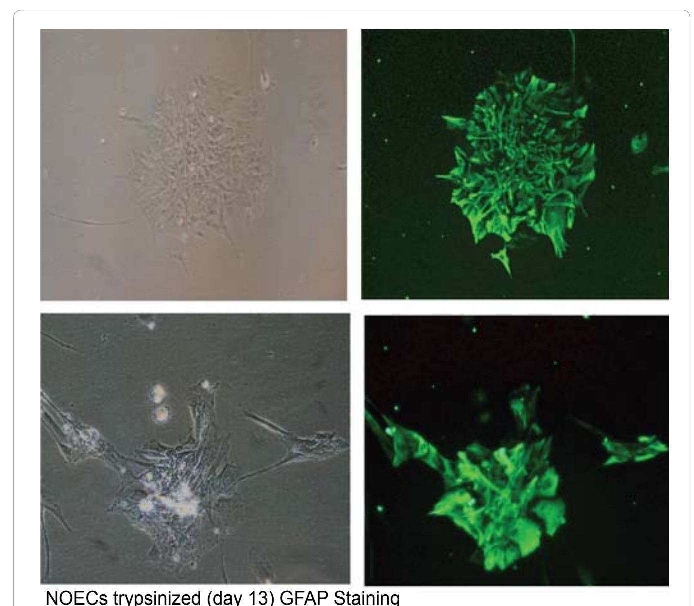
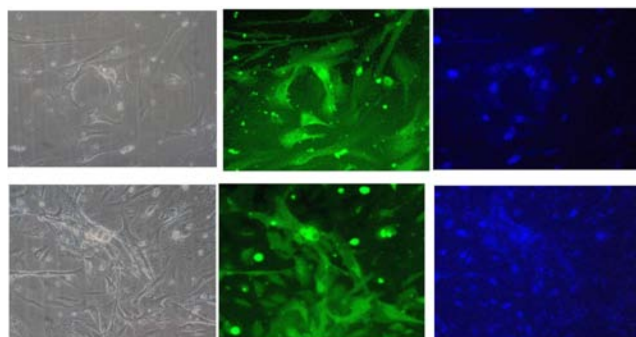
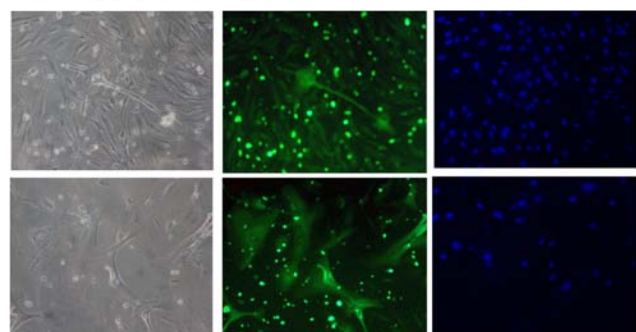


Figure 10: The cell confluency obtained through cultured explants from the neonate rat OB by day 13 was trypsinized. Following trypsination through standard procedures, the cells were microscopically examined and immunologically evaluated after four days of trypsinization. The cells bear a resemblance to those from the parent flask i.e. flat sheet like morphology.



A: HOECs trypsinized (day 21) GFAP Staining



B: HOECs trypsinized (day 21) p75 NTR Staining

Figure 11: The heterogeneous cellular confluency achieved by day 21 through human OM was trypsinized. Following trypsination through trypsin (0.05%), the cells were microscopically examined and immunologically evaluated both through FITC labelled GFAP(A)/p75^{NTR} (B) Protein on 7 days after trypsinization. Cells with flat sheet like morphology showed prominence. The photographs were obtained by Nikon Eclipse Ti Phase Contrast with magnification 10X.

Statistical analysis

The percentage of OECs on each plate was estimated by gross microscopy, rather than direct cell-counting.

Acknowledgement

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