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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/131011> since

Published version:

DOI:10.1016/j.jneumeth.2009.09.021

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Morphological and biomolecular characterization of the neonatal olfactory bulb ensheathing cell line

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Keywords:

Olfactory glia, Neuregulin-1, ErbB receptors, Gene transfer

Abstract

Cell transplantation therapy has raised a great interest in the perspective of its employment for nerve tissue repair. Among the various cell populations proposed, olfactory ensheathing glial cells have raised great interest over recent years, especially in the perspective of their employment for neural repair because of their homing capacity in both central and peripheral nervous system. This paper is aimed to provide an in vitro characterization of the NOBEC (neonatal olfactory bulb ensheathing cell) line that was obtained from primary cells dissociated from rat neonatal olfactory bulb (OB) and immortalized by retroviral transduction of SV40 large T antigen.

Light and electron microscopy investigation showed that NOBECs are a homogeneous cell population both at structural and ultrastructural level. RT-PCR, Western blotting and immunocytochemistry showed that NOBECs express the glial markers S100, GFAP (Glial Fibrillar Acid Protein) and p75NGFR as well as NRG1 (neuregulin-1) and ErbB1-2-3 receptors; while they are negative for ErbB4.

Yet, NOBECs exhibit a high proliferation and migration basal activity and can be transduced with vectors carrying GFP (green fluorescent protein) and NRG1 cDNA. Functional stimulation by means of NRG1-III- β 3 overexpression through viral transduction induced a significant increase in cell proliferation rate while it had no effect on cell migration.

Altogether, these results show that NOBEC cell line retain glial features both morphologically and functionally, responding to the NRG1/ErbB-mediated gliotrophic stimulus, and represents thus a good tool for in vitro assays of glial cell manipulation and for in vivo experimental studies of glial cell transplantation in the central and peripheral nervous system.

1. Introduction

The last 10 years have seen a growing interest in the study of glial cell transplantation for the repair of the damaged nervous system (DeLucia et al., 2003; Geuna et al., 2001; Lavdas et al., 2008; Radtke et al., 2009; Ruitenberg et al., 2006). In this view, the rat olfactory bulb (OB) is of considerable interest as it is the only central nervous system (CNS) tissue that can support continuous neuronal outgrowth throughout the life of the animal (Farbman, 1990; Raisman, 2007). This property has been attributed, at least in part, to the glial cells that reside in the olfactory nerve layer (ONL) of the OB (Raisman, 2007). This specific type of macroglia, termed olfactory ensheathing cells (OECs), shares many properties with Schwann cells but also has characteristics that resemble astrocytes (Doucette, 1995; Sonigra et al., 1999). Ensheathing cells can be purified and cultured *in vitro* comprising only the cells of the nerve fiber layer peeling the outer layer away from the deeper layers of the olfactory bulb. The process to obtain primary OEC cultures provides for chopping into small pieces the dissected nerve fiber layers that are then incubated with collagenase and mechanically disaggregated with a syringe. The resulting cell culture can be maintained in DMEM 10% fetal bovine serum (FBS) (Barnett et al., 2000; Chung et al., 2004; Pellitteri et al., 2009). *In vivo*, these cells guide regenerating axons from the olfactory neuroepithelium into OB by providing a growth-promoting microenvironment. This property of OECs makes them a rational transplantation candidate for therapeutic application not only in the peripheral but also in the central nervous system since, in contrast to transplanted SCs, OECs do not trap regenerating axons in the grafted tissue, possibly because they cause less expression of inhibitory molecules by the host tissue (Lakatos et al., 2000; Raisman, 2007; Radtke et al., 2009). Yet, the transplanted cells have other beneficial effects, such as the promotion of remyelination, secretion of growth factors, vascularization, sparing from secondary damage and enhancement of sprouting, all of which may have an important bearing on functional recovery (Raisman, 2007).

The majority of OECs used for transplant experiment to date has been obtained by primary cultures of OECs (DeLucia et al., 2003). However, preparation of OECs primary cultures has several disadvantages: it is labor-intensive and tedious; the cell population is heterogeneous and cell amount is limited; primary cultures often contain contaminating cells (such as fibroblasts, astrocytes or other cells) and survive only few weeks in culture; they are limited in amount obtainable; animal immunosuppression, that may lead to illness or changes in behavioural recovery, is required for transplantation studies (DeLucia et al., 2003; Moreno-Flores et al., 2006). Moreover, despite the different methods that have been successfully used to purify and transplant OECs, subtle differences in the isolation and preparation of cells mean that no two

laboratories are studying fully comparable cells (Moreno-Flores et al., 2006).

To cope with all these problems, immortalized clonal cell lines can represent a good alternative, at least for experimental animal studies (DeLucia et al., 2003; Lakatos et al., 2000). In 1993, Goodman et al. described a new immortalized cell line – named NOBEC (neonatal olfactory bulb ensheathing cell) – that they derived from neonatal OECs by transduction with SV40 large T cell antigen (Goodman et al., 1993). These authors demonstrated that NOBECs are immortal but minimally transformed, in fact they maintain viable monolayers, do not form tumors when transplanted into rats and have a low proliferation rate in comparison to glioma cells. However, immortalization can lead to subtle alteration of cell properties and functional activity and thus in-depth in vitro morphological and functional characterization of a cell line is an important prerequisite before its in vivo employment for cell transplantation studies. Therefore, in order to provide a further characterization of the NOBECs, in the present study we asked four questions:

- (1) Are NOBECs a homogenous cell population at structural and ultrastructural level?
- (2) Do NOBECs express Schwann cell markers (S100 and GFAP) as well as the components of the NRG1-ErbB gliotrophic system?
- (3) Which is the basic proliferation and migration rate of NOBECs in comparison to other cell lines?
- (4) Can NOBECs be efficiently manipulated by viral gene transfer and how do they react when the glial growth factor NRG1 is overexpressed?

2. Materials and methods

2.1. Cell cultures

The NOBEC cell line was kindly provided by Dr. Jacobberger (Comprehensive Cancer Center, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 4106-4944, USA). The NOBEC cell line was derived from primary cells dissociated from neonatal rat olfactory bulb and immortalized by retroviral transduction of SV40 large T antigen (Goodman et al., 1993).

NOBECs were grown in monolayer at 37 ° C in a humidified atmosphere of 5% CO₂/air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). Subconfluent cells were routinely harvested by trypsinization and seeded in 57-cm² dishes (1 × 10⁶ cells).

NIH3T3 and ST14A cell lines were maintained as recommended by American Type Culture Collection and as described by Cattaneo and Conti (1998), respectively.

2.2. Gene transfer

2.2.1. Subcloning and expression of GFP (NOBEC GFP cells)

To generate stable cell clones expressing the green fluorescent protein (GFP), an EcoRI-NotI fragment, containing the entire GFP cDNA, was subcloned into the pIRESpuro2 vector (CLONTECH Laboratories) EcoRI and NotI sites.

For stable transfection, growing NOBECs (10 cm diameter plate, 60% confluent) were transfected overnight with 10 mg DNA using 20 ml LipofectAMINE (Invitrogen) and Opti-MEM I (Invitrogen), according to the manufacturer's recommendations. DNA was prepared using the GenElute™ endotoxin-free plasmid maxiprep kit (Sigma). After 60 h incubation in the appropriate growth medium, the clone selection was carried out for 2 weeks in 5 mg/ml puromycin. Individual clones were screened for their GFP auto-fluorescence and recovered.

2.2.2. Cloning and expression of neuregulin-1-typeIII-β3 (NOBEC-NRG1 cells)

Total RNA was prepared from olfactory bulbs obtained from one adult Wistar rat (Charles River Laboratories) using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 mg) was subsequently reverse transcribed to cDNA as previously described (Gambarotta et al., 2004).

Rat neuregulin-1-typeIII-β3 (NRG1-III-β3) cDNA was cloned by nested PCR amplification following the cloning strategy described for the human NRG1-III-β3 cDNA (Schroering and Carey, 1998). PCRs were carried out in a total volume of 50 μl containing 5 μl cDNA, 250 nM each

forward and reverse primers, 1× buffer, 2.5 units PfuTurbo[®] DNA polymerase (Stratagene), 100 mM dNTPs, and 5% glycerol.

To choose primers, the human NRG1-III-β3 cDNA was compared with rat NRG1 sequences; the primers (produced by Sigma-Genosys) were designed according to the GenBankTM/EBI rat NRG1-typeIII-b1a (accession number AF194438) and rat neurotrophin-3 differentiation factor/NDNF (accession number U02315). The primers for the “first round” are: forward 5′ - AGATGCTGTATCATTGGTTGGGG-3′, and reverse, 5′-GTTCTCATGCGACAGGCAGAGG-3′; for the “second round” are: forward, 5′ -ctcGagGGTgGgCGATGGAGATTTATTCC-3′, and reverse, 5′ -ctgcAGGGGAG**ATGT**AGCAACAAGAAAGC-3′, with the artificial XhoI and PstI sites underlined and the ATG codon in boldface. Small case letters indicate nucleotides different from the wild type sequence.

First round PCR was performed following a simplified “touch-down” protocol: denaturation at 95°C for 5 min; followed by 10 cycles of denaturation at 95°C for 30 s, annealing at 67°C for 30 s, and extension at 72°C for 3 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 3 min.

For the second round PCR, 1 ml of first round PCR product was re-amplified through 30 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 3 min.

PCR products were cloned into the pCR[®]-BluntII TOPO[®] vector (Invitrogen) using the Zero Blunt[®] TOPO[®] PCR cloning kit (Invitrogen) following the manufacturer’s instructions.

For each clone, the orientation of the cDNA in the vector (sense or antisense) was detected by restriction enzyme digestions using XhoI (Amersham Biosciences).

Two cDNA clones corresponding to the full-length rat NRG1-III-β3 isoform were completely sequenced on both strands (Genelab Laboratories, Rome, Italy) and the sequence has been submitted to the GenBankTM/EBI Data Bank with accession number DQ176766.

To express NRG1-III-β3, the cDNA was subcloned into the lentiviral expression vector MA1 (Amendola et al., 2005).

MA1 has been kindly provided by prof. Luigi Naldini (San Raffaele Telethon Institute for Gene Therapy, Milano, Italy). This vector is endowed with a bidirectional promoter driving both neuronal growth factor receptor (NGFR) and GFP transcription. In place of NGFR cDNA we inserted NRG1-III-β3 cDNA.

NRG1-III-β3 cDNA was excised from pCR[®]-BluntII TOPO[®] vector by digestion with EcoRI, and subcloned into the MA1 lentiviral vector previously digested with SmaI and Sall (to extract NGFR cDNA). EcoRI and Sall cohesive ends have been blunt ended to allow ligation.

The orientation of the cDNA inside the lentiviral vector was detected by BamHI restriction

enzyme digestion.

Transient transfection of COS cells with MA1-NRG1-III- β 3 construct has been performed to verify, by Western blot, the ability of this vector to afford in vitro the expression of the NRG1 protein. Cells were transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Lentivirus has been produced and titrated according to the protocol described by Amendola et al. (2005).

In order to verify that the observed results are due to the overexpression of NRG1-III- β 3 and not to the lentiviral infection, a second approach to express NRG1-III- β 3 was used. NRG1-III- β 3 cDNA was subcloned into the adeno-associated-virus vector (pAAV-MCS, Stratagene, LA Jolla, CA) kindly provided by Prof. Mauro Giacca (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy)

NRG1-III- β 3 cDNA was excised from pCR[®]-BluntII-TOPO[®] vector by digestion with EcoRI, and subcloned into the MA1 adeno-associated viral vector previously digested with Sall; EcoRI and Sall cohesive ends have been blunt ended to allow ligation. The orientation of the cDNA inside the pAAV-MCS vector was detected by BamHI restriction enzyme digestion.

For transient transfection, growing NOBECs (10 cm diameter plate, 60% confluent) were transfected over night with 10 mg DNA (pAAV-NRG1-III- β 3) using 20 ml LipofectAMINE (Invitrogen) and Opti-MEM I (Invitrogen), according to the manufacturer's recommendations.

48 h after transient transfection, RNA and proteins have been extracted and analyzed as described.

2.3. GFP transfection efficiency

NOBEC GFP and NOBEC-NRG1-LV cells were cultured at 37°C with 5% CO₂ for 2 days. The cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min and then stained with 4',6-diamidino-2-phenylindole (DAPI) 0.2 mg/ml for 10 min. Pictures of fixed cells were acquired using an inverted microscope (Olympus Corp 1X50) equipped with a charge-coupled device camera CoolSNAP-Pro (Media Cybernetics).

The efficiency of GFP transfection was quantified by measuring the percentage of GFP cells observable in each picture compared with the nuclei stained with DAPI staining.

Briefly, nine images were captured for each dish. Photos were taken randomly using a 10x objective. The images were edited with Image J software. The Image J plugin cell counter was then used to evaluate the cell number.

Data obtained from three dishes for both NOBEC GFP and NOBEC-NRG1-LV samples, were analyzed, averaged, and expressed as mean \pm standard deviation.

2.4. *Light and electron microscopy*

To investigate adherent NOBECs, cells were cultured on Lab-Tek chamber slide (Sigma, St. Louis, MO). To investigate NOBECs in suspension, cells were detached from the dish by incubation with 0.25% trypsin and 0.1% EDTA for 5–10 min at 37 ° C. Complete medium was added to inactivate the trypsin and one drop of medium containing glial cells was put on a slide.

For light microscope observation, some slides were stained with haematoxylin and eosin.

For electron microscopy, NOBECs were centrifuged at 900 × *g* for 5 min and the pellet was fixed in 1% paraformaldehyde (Merck, Darmstadt, Germany), 1.25% glutaraldehyde (Fluka, St. Louis, MO, USA) and 0.5% saccharose in 0.1 M Sörensen phosphate buffer (pH 7.2) for 2 h. The pellet was then washed in 1.5% saccharose in 0.1 M Sörensen phosphate buffer (pH 7.2) for 6–12 h, postfixed in 2% osmium tetroxide, dehydrated and embedded in Glauert's embedding mixture, which consists of equal parts of Araldite M and Araldite Härter, HY 964 (Merck, Darmstadt, Germany), supplemented with 2% of the accelerator DY 064 (Merck, Darmstadt, Germany). The plasticizer dibutyl phthalate was added at 0.5%. Thin sections (70 nm) were cut using a Leica Ultracut UCT, stained with uranyl acetate and lead citrate and examined in a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for computerized acquisition of images.

2.5. *Immunocytochemistry and confocal microscopy*

For imaging of the GFP auto-fluorescence of the NOBECs, unstained slides were directly analyzed by fluorescence microscopy. For immunocytochemistry, some slides were then incubated with primary antibody against ErbB1 (policlonal, rabbit, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), ErbB2 (policlonal, rabbit, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), ErbB3 (policlonal, rabbit, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), ErbB4 (policlonal, rabbit, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), GFAP (glial fibrillar acid protein, monoclonal, mouse, 1:70, Dako Italia, Milano), S100 (policlonal, rabbit, 1:600, Sigma, St. Louis, MD, USA), p75NGFR (monoclonal, rabbit, 1:50, Abcam, Cambridge, UK). After washing in PBS, sections were then incubated with CY3 secondary antibody (a-rabbit IgG, Jackson immunoresearch Europe, Suffolk UK). For specificity assessment, all antibodies were checked by immunocytochemistry secondary antibody labelling with omission of the primary antibody, which led to no immunopositivity.

Confocal imaging was carried out with a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany), which incorporates two lasers (Argon and HeNe) and is equipped with an

inverted Axiovert 100 M microscope. Confocal fluorescence images were taken using a 20× Plan-NEOFLUAR objective with a numerical aperture (NA) of 0.50 and a 40× Plan-NEOFLUAR objective with a NA of 0.75. An electronic zoom with a magnification ranging from 1 to 8 was employed to obtain the magnifications indicated in the figures. To visualize CY3 fluorescence, we used excitation from 543-nm HeNe laser line and emission passing through a high-pass (LP) 560 filter which passes wavelengths superior to 560 nm to the detector. Images created with the BP 505–530 filter were digitally colored green. Images created with the LP 560 filter were digitally colored red.

2.6. RT-PCR

Total RNA was isolated by extraction with TRIzol (Invitrogen) and digested with 1 U/mg DNaseI (RNase free) at 37°C for 30 min (Promega). DNase I was then heat-inactivated at 65°C for 10 min.

RNA yield was quantified by measuring absorbance at 260/280 nm. Total RNA (500 ng per each sample) was reverse transcribed in a reaction volume of 20 µl with 50 pmol of random hexamers (Amersham Pharmacia). Each reaction consisted of cDNA synthesis buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 1 mM dNTP (Amersham Biosciences), 1.8 U/ml RNAsin (Amersham Biosciences) and 10 U/ml M-MLV reverse transcriptase (Amersham Biosciences). Samples were then incubated at 37°C for 1 h.

PCR reactions were carried out in a total volume of 30 µl including 2 µl of reverse transcribed RNA, the appropriate oligonucleotides (0.6–6 mM), standard reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin) (Sigma-Aldrich), 0.8 mM dNTP (Amersham Biosciences) and 0.025 U/ml of REDTaq DNA polymerase (Sigma-Aldrich). The REDTaq DNA polymerase was added last, after the samples were heated to 95°C.

Specific primers designed to amplify ErbB1, ErbB2, ErbB3, ErbB4 (which amplify cyt1 and cyt2 ErbB4 isoforms), NRG1, p75NGFR, GFAP, S100 and G3PDH are listed in Table 1. G3PDH amplification was used to monitor the quality and quantity of RNA that had been reverse transcribed into cDNA. For NRG1, distinct specific pair of primers were used (Cote et al., 2005). One primer (forward) was designed to the NRG1 Ig domain (common to types I and II NRG1) and either (reverse) the a or b domains, whereas the other set of primers allows the amplification of all type III isoforms of NRG1. Samples were amplified for 25–33 cycles, depending on the primer pair (Table 1). Cycling parameters were set for each primer pair in order to obtain specific products in the exponential amplification phase of the PCR reaction. Amplification products were separated by agarose gel electrophoresis and DNA bands visualized with ethidium bromide staining (2 ×

10⁻⁵%). Negative controls were performed from reverse transcription reactions lacking reverse transcriptase.

The number of cycles and the annealing temperature used for each primer pair were: 33 cycles and 60°C for NRG1 and GFAP, 30 cycles and 60°C for ErbB receptors, 28 cycles and 60°C for S100, 25 cycles and 60°C for G3PDH.

2.7. Western blotting

Total proteins were solubilized in lysis buffer (Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.5% NaDOC, 1% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 2 mM orthovanadate) on ice. Protein content was determined using a bicinchoninic acid kit for protein determination (Sigma-Aldrich). Protein extracts (30 mg/lane) were subjected to 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto Hybond membrane (Amersham Biosciences) according to the manufacturer instructions. After blocking with 5% powder milk in TBST buffer (20 mM Tris; 150 mM NaCl; 0.1% Tween 20, pH 7.4), filters were probed with the specific antibodies diluted 1:500 in TBST buffer containing 1% no-fat powder milk. Primary antibodies were visualized with the appropriate peroxidase-coupled secondary antibodies by using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

The polyclonal antibodies used for ErbB receptors (purchased from Santa Cruz) are: a-ErbB1 (catalog no. sc-003), a-ErbB2 (catalog no. sc-284), a-ErbB3 (catalog no. sc-285), a-ErbB4 (catalog no. sc-283); while to detect NRG1-III-β3 protein the a-NRG1-III-β3 N-term (catalog no. AB-551) purchased from Chemicon was used.

2.8. Proliferation assay

To determine proliferation, cells were plated at a concentration of 3 × 10⁴ cells/3-cm dish. After 2 h in 10% serum, cells were starved for 96 h in medium with 2% FBS. After 24–48–72 and 96 h, cells were trypsinized and counted in a Burker's hemocytometer chamber. For each time point, four dishes were used and the experiments were performed in triplicates. All counts obtained from assays were analyzed, averaged, and expressed as mean ± standard deviation and Student-Newman-Keuls tests were used to compare data from different groups.

2.9. Migration assay

The Transwell migration assay was used to measure three-dimensional movement. Cells (10⁵) were seeded on the upper side of a Falcon cell culture insert (Becton Dickinson Labware, Franklin Lakes, NJ) on a porous polycarbonate membrane (8 mm pore size, 1 × 10⁵ pores/cm²); the lower

chamber of the 24 multiwell cell plate was filled with medium containing 2% FBS. After 6 h of incubation, cells attached to the upper side of the filter were mechanically removed, whereas cells that migrated to the lower side of the filter were fixed in PBS containing 2% glutaraldehyde. Cells were then stained by addition of 500 μ l/well of a solution containing 0.1% crystal violet in 20% methanol. After being shaken (200 cycles/min) for 20 min at room temperature, plates were washed five times by submersion in deionised water and air dried at least 24 h and then the bound dye was solubilized by addition of 100 μ l/well of 10% acetic acid and 5 min shaking at room temperature. The OD of dye extracts was measured directly in plates using a Microplate Reader (Bio-Rad, Hercules, CA), at the wavelength of 590 nm.

For each cell line, nine wells were used and the experiments were performed in triplicates. Results from three different experiments were averaged and expressed as means \pm S.D. ANOVA and Student–Newman–Keuls tests were used to compare data from different groups.

3. Results

3.1. *Light and electron microscopy characterization*

After haematoxylin and eosin staining (Fig. 1A and B), NOBECs showed a rather homogenous appearance with a round shape, a basophilic and central nucleus and an eosinophilic cytoplasm. The plasma membrane showed a regular profile that was detectable also at electron microscopy observation (Fig. 1C and D). Morphological homogeneity was confirmed at ultrastructural level where NOBECs showed a central and irregularly shaped nucleus with one or more nucleoli (arrows in Fig. 1C and D). Chromatin was spread throughout the nucleus except for a thin dense layer located immediately inside the perinuclear cisterna (arrow in Fig. 1E).

Organelles showed a uniform distribution throughout the cytoplasm. Mitochondria showed elongated shape with light matrices and thin cristae (Fig. 1F). Endoplasmic reticulum was mainly granular and organized into small and dilated elements (Fig. 1E and F) which gave to the cytoplasm a vacuolated appearance. The cytoplasmic matrices are rich of free ribosomes (arrows in Fig. 1F). When these cells are adherent to the plate, they have a polygonal shape and growth forming clusters (Fig. 2).

We have also observed that the transduction with lentiviral vector does not modify the morphology of NOBECs (Fig. 3). In fact, infected NOBECs maintained a polygonal shape with a central nucleus. As expected, lentiviral transduction resulted in a strong green fluorescence and the efficiency of cells infection is more than 80%.

3.2. *mRNA and protein expression in NOBEC*

We examined the expression of glial markers and NRG1/ErbB system in the NOBEC cell line by RT-PCR. The positive control was the cDNA obtained from rat olfactory bulb. Results (Fig. 4A) showed that NOBECs express messengers encoding for p75NGFR, GFAP and S100. The RT-PCR analysis for the expression of transcripts of ErbB receptors showed that of the four known ErbB kinases, the cell line expresses ErbB1, ErbB2 and ErbB3 receptors. The expression of the three ErbB receptors and glial markers is also confirmed at protein level by Western blotting (Fig. 4B) and immunocytochemical analysis (Fig. 4B). Fig. 4B shows adherent NOBECs immunopositive for three receptors of the ErbB family ErbB1, ErbB2, ErbB3, and also for three glial markers GFAP, S100 and p75NGFR. NOBECs are negative for ErbB4.

To investigate the presence of mRNA coding for NRG1, we have employed different pairs of primers that allow the amplification of different NRG1 isoforms. Results of RT-PCR analysis (Fig. 4C) revealed that the cell line expresses different NRG1 isoforms. NOBECs express at least one

immunoglobulin NRG1 (Ig-NRG1) with an a EGF-like domain and at least one Ig-NRG1 with a b EGF-like domain. The immunoglobulin domain is common to all type I NRG1 and some type II NRG1. Moreover, to determinate if type III NRG1 were present, we screened RNA by RT-PCR with cysteine-rich domain primers. Both isoforms (typeIII-NRG1a and typeIII-NRG1b) are expressed in the cell line.

In a second investigation step, the expression of S100/GFAP glial markers and of NRG1/ErbB system was evaluated in NOBECs expressing GFP (NOBEC GFP) as well as in NOBECs overexpressing NRG1 by two different viral-mediated gene transfer approaches: lentivirus (NOBEC-NRG1-LV) and adeno-associated-virus (NOBEC-NRG1-AAV). Messenger analysis showed that all NOBECs have the same expression profile concerning both glial markers and the three receptor of ErbB family (ErbB1, ErbB2 and ErbB3) (Fig. 5A).

As regards the expression of the different isoforms of NRG1 we observed, as expected, a strong up-regulation of NRG1-typeIII-b and, specifically, NRG1-typeIII-b3, in NOBECs carrying the vector expressing NRG1-typeIII-b3, independently of the type of viral vector adopted for gene transfer. Notably, the expression of Ig-NRG1 and NRG1-typeIII-a was down-regulated. This down-regulation is stronger following lentiviral infection (Fig. 5B). The overexpression of the NRG1-typeIII-b3 in genetically manipulated NOBECs was also confirmed at protein level by Western blotting (Fig. 6).

3.3. Proliferation rate of the NOBEC line

The proliferation ability of NOBECs was evaluated by curve analysis. We compared the proliferation ratio of NOBECs with two different cell lines known to have a different proliferation and migration activity: ST14A (cell line that was derived from embryonic day E14 rat striatum primordia by retroviral transduction of the temperature-sensitive SV40 Large T Antigen; Cattaneo and Conti, 1998) which have higher proliferation and lower migration rate, and NIH3T3 (cell line that has been developed from NIH Swiss mouse embryo cultures; ATCC number CRL-1658) which have lower proliferation and higher migration rate. In addition, in order to evaluate the ability of NRG1-typeIII-b3 to promote cell proliferation we analyzed the proliferation ratio of NOBECs after NRG1-typeIII-b gene transfer.

Fig. 7A and B show the variations in cell number of the four cell lines that were starved for 96 h in medium with 2% FBS. Statistical comparison exhibit that, as expected, at 96 h NIH3T3 proliferate less than ST14A ($p < 0.05$) while NOBECs proliferation activity, though present, was significantly ($p < 0.05$) lower than both cell populations. Concerning NOBEC-NRG1-LV cells, proliferation was significantly higher than both NOBEC and NIH3T3 populations and comparable

to the ST14A cells.

3.4. Three-dimensional migration assay of NOBEC line

In order to evaluate the ability of NOBECs to migrate in a three-dimensional environment, we assayed basal motility using Transwell assay performed with NOBECs, NOBEC-NRG1-LV, ST14A and NIH3T3 in 2% FBS. The quantitative analysis of migrated cells after 6 h of incubation showed, as expected, the significantly ($p < 0.05$) different migratory activity between ST14A and NIH3T3 cell line. NOBECs exhibited a significant higher basal migration activity when compared with both ST14A ($p < 0.01$) and NIH3T3 ($p < 0.05$) (Fig. 7C). Up-regulation of NRG1-typeIII-b3 did not induce a significant effect on NOBEC migration.

4. Discussion

The potentiality of OEC transplantation for enhancing neural regenerative process after damage has opened new perspectives for the treatment of severe neural injuries (Dombrowski et al., 2006; Novikova et al., 2006; Radtke et al., 2009; Raisman, 2007). In fact, among the various potential sources of cells, OECs have been extensively studied because they represent a unique type of macroglia required for normal olfactory axonal regeneration throughout the life time of an individual providing a growth-promoting microenvironment (De Mello et al., 2007; Dombrowski et al., 2006). Unfortunately, the number of OECs that can realistically be harvested from each subject is limited and ensuring a pure cell population is difficult. The generation of immortalized OEC lines constitutes thus a rational approach to obtain a sufficient amount of cells retaining the axonal regeneration-promoting properties of primary OEC cultures.

A particularly interesting cell population is represented by a clonal cell line of OECs that have been obtained by immortalization with viral oncogene SV40 large T antigen (Goodman et al., 1993). In order to increase basic biological knowledge on the morphological and functional properties of this cell line, the aim of the present study was to further characterize NOBECs in order to give an answer to the following four questions.

4.1. Are NOBECs a homogenous cell population at structural and ultrastructural level?

Light and electron microscope observations reported in the present paper show that NOBECs, both in culture and after suspension, are a uniform cell population with ultrastructural features of relatively immature cells (round shape, large central nucleus and little eosinophilic cytoplasm with few organelles uniformly distributed). Notably, the ultrastructure of NOBECs was not modified by viral-mediated gene transfer in agreement with previous observations in other cultured stem cell populations (Raimondo et al., 2006). Although our morphological observations support the view that NOBECs are a rather pure cell line, FACS analysis for a panel of surface markers might be useful to further explore purity of this cell line and, in case of the identification of subpopulations, it might allow separation of the group of cells which is more suitable in the perspective of in vivo transplantation for neural repair.

4.2. Do NOBECs express Schwann cell markers (S100 and GFAP) as well as the components of the NRG1-ErbB gliotrophic system?

Despite their immature morphological features, we found that NOBECs express main glial proteins GFAP and S100 that are expressed in primary OEC cultures (DeLucia et al., 2003; Goodman et al., 1993) and p75NGFR, a glial marker that is useful not only to confirm the glial

nature of NOBECs, but also to assess the purity of this cell line (Wu et al., 2009). In particular, cell counting showed that all NOBECs are immunolabelled by the α -p75NGFR antibody.

As a further demonstration of the maintained glial nature of the NOBEC line, we also found that they express both neuregulins and their ErbB receptors. Primary cultures of OECs, Schwann cells and astrocytes express different combination of ErbB receptors through which they respond to the many NRG1 isoforms that are secreted in their environment (Pollock et al., 1999; Thompson et al., 2000). Pollock et al. (1999) have described that primary cultures of OECs are positive for ErbB2 and ErbB4 but negative for ErbB3 in immuno-cytochemical staining. In contrast, immunohistochemical studies have reported the expression in vivo of ErbB3 by non-neuronal cells of the olfactory nerve (Perroteau et al., 1999). In more recent study on primary and immortalized OECs (Moreno-Flores et al., 2003) it has been shown, by Western blot analysis, that primary OECs express ErbB2, and low levels of ErbB4 and ErbB3, indicating that the failure to detect this receptor previously in cultured OECs was due to the lower sensitivity of the applied technique.

4.3. Which is the basic proliferation and migration rate of NOBECs in comparison to other cell lines?

Our results showed that NOBECs exhibited a low basal proliferation activity while they have a high migratory capacity. The migration capacity is particularly important in the light of the employment of NOBECs for transplantation studies since it will permit transplanted cells to move from the site of release to the sites of damage where they can contribute to the repair process.

4.4. Can NOBECs be efficiently manipulated by viral gene transfer and how do they react when the glial growth factor NRG1 is overexpressed?

Several studies have demonstrated that NRG1-ErbB system is strongly involved in Schwann cell-axon communication during developmental stages and following nerve injuries (Britsch, 2007; Geuna et al., 2007; Nave et al., 2007; Raimondo et al., 2005). NRG1 is expressed not only by axons but also by astrocytes, Schwann cells and oligodendrocytes (Raabe et al., 1997) and particularly NRG b isoform seems to be involved in Schwann cell proliferation and Schwann cell precursor survival (Reinhard et al., 2008). Among the different NRG1 b isoforms, recently much attention has been paid to NRG1-typeIII-b3, that elicit its biological activity on both proliferation of SC precursors and myelination (Nave and Salzer, 2006; Taveggia et al., 2005). Based on these evidences, we have infected NOBECs cells with a lentiviral vector expressing both GFP and NRG1-typeIII-b3. RT-PCR analysis confirmed that infected NOBECs have the same expression

profile of wild type NOBECs as regard glial proteins (GFAP and S100) and ErbB receptors. Green fluorescence is evident and durable, an important feature in the light of transplantation experiments (Ramer et al., 2004).

As regards NRG expression, we showed that viral-mediated overexpression of NRG1-typeIII-b3 induced a significant down-regulation of NRG1 types I, II and III-a. One possible explanation of the observed down-regulation of various NRG1 isoforms is the existence of a compensatory mechanism consequent to the up-regulation of the type III-b3 specific isoform.

Finally, data obtained in our functional experiments demonstrated that NRG1-typeIII-b3 is able to enhance proliferation of NOBECs, while its effects on cell migration capacity were not statistically significant, confirming the mitogenic effect of this ligand (Thompson et al., 2000).

In conclusion, results of this study show that NOBECs retained glial features both under basal conditions and after in vitro manipulation by viral-mediated gene transfer with NRG1, and represent thus a good tool for in vitro assays of olfactory glial cell manipulation as well as for in vivo experimental studies of olfactory glial cell transplantation in the central and peripheral nervous system.

Acknowledgements

The authors are grateful to Dr. Jacobberger (Comprehensive Cancer Center, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 4106-4944, USA) for providing the NOBEC cell line.

The authors wish to thank Prof. Luigi Naldini for providing the MA1 lentiviral vector, Prof. Mauro Giacca for providing the pAAV-MCS vector. The authors also wish to thank Prof. Roberto Piva and Dr. Chiara Ambrogio for providing facilities and expert assistance for lentiviral vector manipulation. This work was supported by grants from the Regione Piemonte (“Bando Ricerca Sanitaria Finalizzata”), the Ministero Italiano dell’Università e della Ricerca (MIUR), FIRB fund (code: RBAU01BJ95), PRIN2005 fund (code: 2005057088), the University of Turin (ex-60% fund), and the Compagnia di San Paolo (“Bando Programma Neuroscienze”). Dr. Stefania Raimondo is recipient of a PostDoc grant partially funded by the Regione Piemonte (“Azione Contenimento del Brain Drain”).

Figure legends

Fig. 1. (A and B) Haematoxylin and eosin staining of suspended NOBECs. All cultured cells showed a basophilic and central nucleus and an eosinophilic cytoplasm. (C–F) Electron microscopy images of NOBECs. All cells showed similar morphological features: a central and irregular shaped nucleus with some nucleoli (arrows in C and D), a rich granular endoplasmic reticulum and many free ribosomes (arrows in F). Scale bars (A–D): 5 μm ; (E): 0.5 μm ; (F): 1 μm .

Fig. 2. Haematoxylin and eosin staining of adherent NOBECs. All cultured cells showed a polygonal shape and growth forming clusters. Scale bars (A and B): 20 μm .

Fig. 3. Images of adherent NOBEC-NRG1-LV cells. (A) Phase contrast; (B) green fluorescence; (C) merging. Cells showed similar morphology in comparison to NOBEC wild type. The presence of fluorescent cells confirms the success of lentiviral infection. Original magnification. Scale bars: 10 μm .

Fig. 4. (A) RT-PCR expression profiles of glial markers (GFAP, S100 and p75NGFR) and ErbB receptor messengers in NOBECs in comparison to cDNA obtained from rat OB. G3PDH served as control. (B) Western blotting and immunocytochemical analysis of glial markers and ErbB receptor proteins. (C) RT-PCR analysis of the NRG1 isoforms. Scale bars: 10 μm .

Fig. 5. (A) RT-PCR expression profiles of glial markers (GFAP and S100) and ErbB receptor messengers in NOBEC-NRG1-LV in comparison to NOBEC, NOBEC GFP and NOBEC-NRG1-AAV cells. G3PDH served as control. (B) RT-PCR analysis of the NRG1 isoforms.

Fig. 6. Western blotting analysis of NRG1-III- β 3 protein in NOBEC and NOBEC-NRG1-LV cells. COS cells transfected or not by NRG1-III- β 3 are used respectively as positive and negative controls. The 40 kDa NRG1-III- β 3 isoform is recognized by aNRG1 type III antibody. The other bands are non-specific, being recognized by aNRG1 type III primary antibody in whole cell protein extracts.

Fig. 7. Proliferation assay (A and B) and migration assay (C) of NOBEC, NOBEC-NRG1-LV and two different cell lines used as controls (ST14A and NIH3T3) in medium with 2% FBS. NOBECs exhibited a remarkable proliferation activity, even if significantly lower compared with the other

two cell lines, and a high basal migration activity in a three-dimensional environment; while NOBEC-NRG1-LV have both high proliferation and migration activities. Asterisks indicate statistical significance in respect to NOBEC (B) or ST14A (C) (*p < 0.05 and **p < 0.001). Hash key indicate statistical significance in respect to NIH3T3 (B and C) or (# p < 0.05).

Gene	Primer sequence		Accession number and product size
GFAP	5' -TGGACCGATACCACTCTTCTGT-3'	Forward	NM 017009, 321 bp
	5' -GAAGCTCCAAGATGAAACCAAC-3'	Reverse	
S100	5' -CTGTGGGTATAGCACTGGTTGTAG-3'	Forward	NM 013191, 242 bp
	5' -AGGGTGTGGGTGATAGGTCTAACT-3'	Reverse	
P75NGFR	5' -GTGGAACAGCTGCAAACAAA-3'	Forward	NM 012610, 223 bp
	5' -CTCTACCTCCTCACGCTTGG-3'	Reverse	
ErbB1	5' -TATAGCTCCGATCCCACCAG-3'	Forward	U52529, 210 bp
	5' -ATACTCAGGGTTGCTCAGCG-3'	Reverse	
ErbB2	5' -ATCCTAATGAAACGAAGGAGACAG-3'	Forward	NM 017003, 263 bp
	5' -GATGTGTTTTCTCTCAACACCTTG-3'	Reverse	
ErbB3	5' -CTGTTTAGGCCAAGCAGAGG-3'	Forward	NM 017218.2, 208 bp
	5' -GACTTTGTTTGCCTTCTCGC-3'	Reverse	
ErbB4	5' -TGCTGAGGAATATTTGGTCCCCCA-3'	Forward	L47241, 216 bp (cyt1), 168 bp (cyt2)
	5' -TCTGGTATGGTGCTGGTTGTGGCT-3'	Reverse	
NRG1		Immunoglobulin	
	5' -CAGAAGAAGCCAGGGAAGTC-3'	domain (Ig) forward	RNU02323 for Ig-NRG1a(392 bp)
		Cysteine-rich domain	
	5' -TGGCAACGATCACCAGTAAA-3'	(typeIII) forward	RNU02322 for Ig-NRG1b(401 bp)
	5' -GCTCCAGTGAATCCAGGTTG-3'	EGF-a reverse	AF194439 for TypeIII-NRG1a(207 bp)
G3PDH	5' -TGGCATTGTGGAAGGGCTCATGAC-3'	Forward	M32599, 188 bp
	5' -ATGCCAGTGAGCTTCCCGTTCAGC-3'	Reverse	

Table 1
Primers used for RT-PCR analysis.

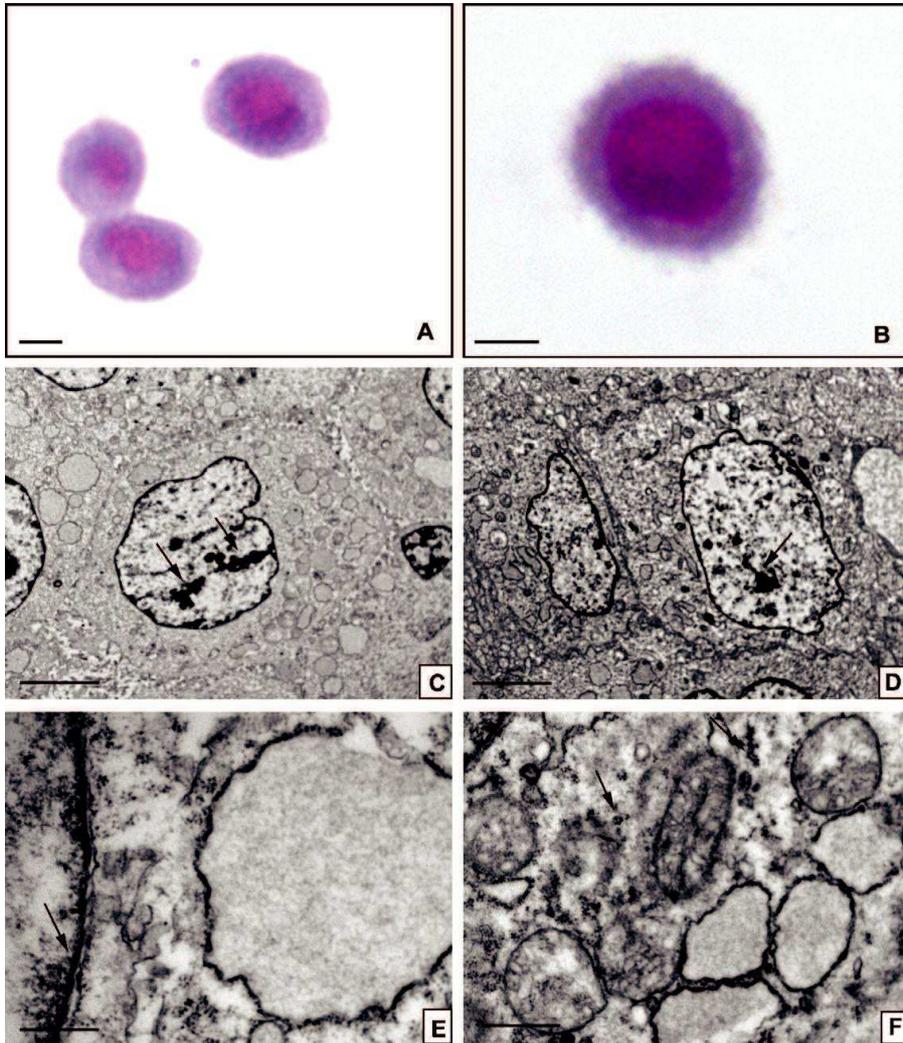


Figure 1

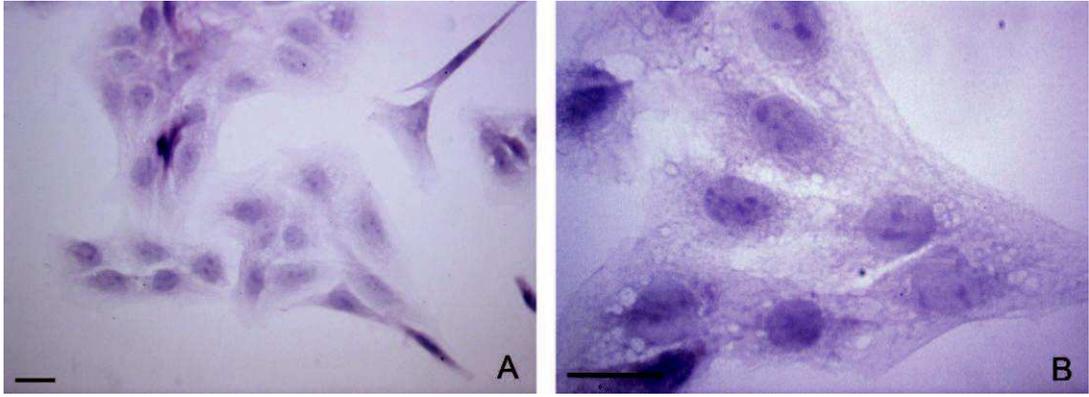


Figure 2

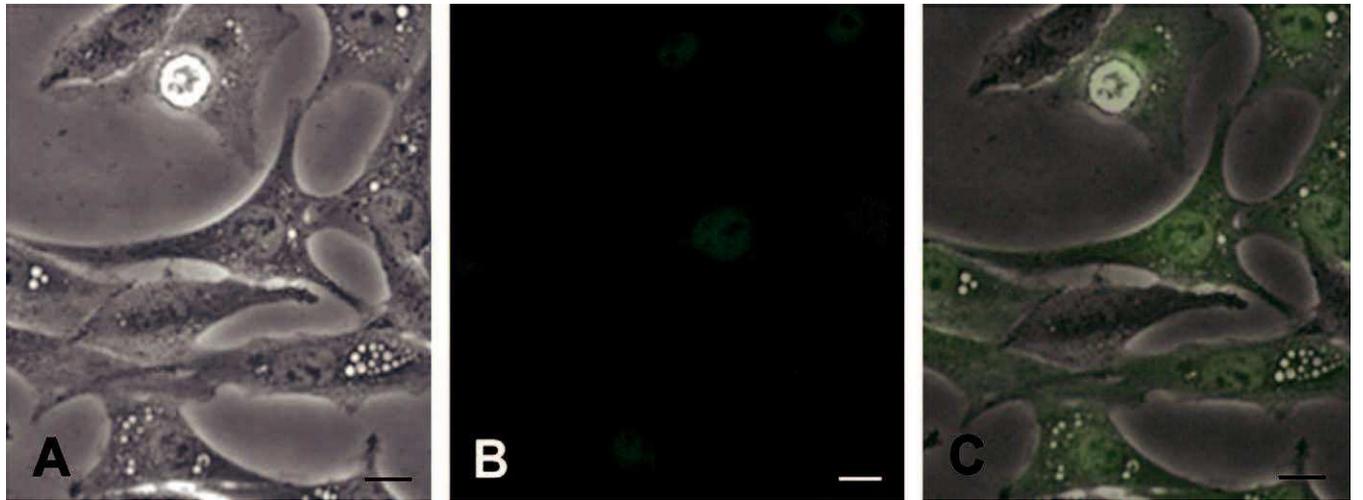
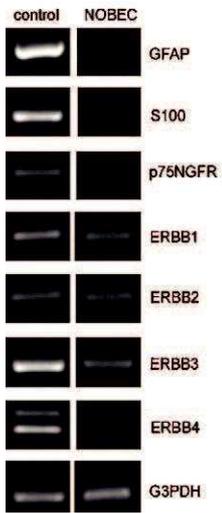
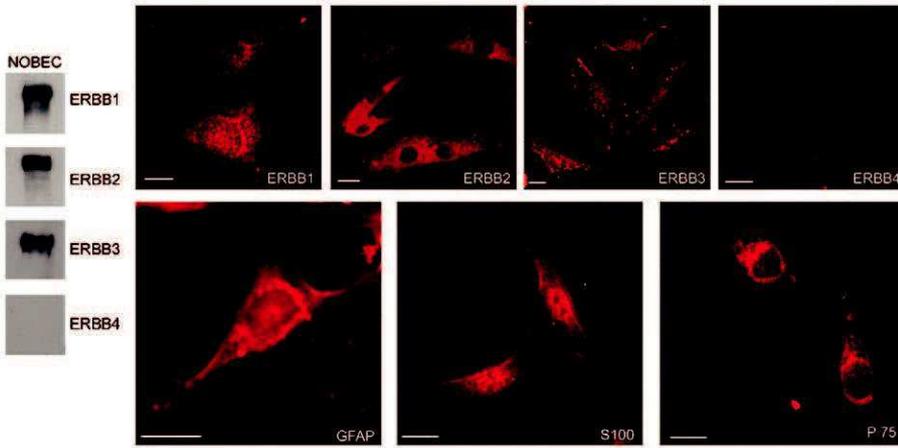


Figure 3

(A) mRNA expression



(B) protein expression



(C) NRG1 isoforms mRNA expression

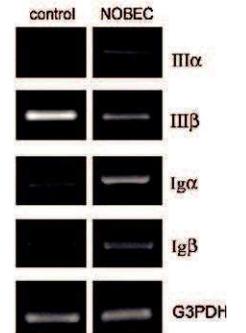


Figure 4

(A) glial markers and ErbB mRNA expression (B) NRG1 mRNA expression

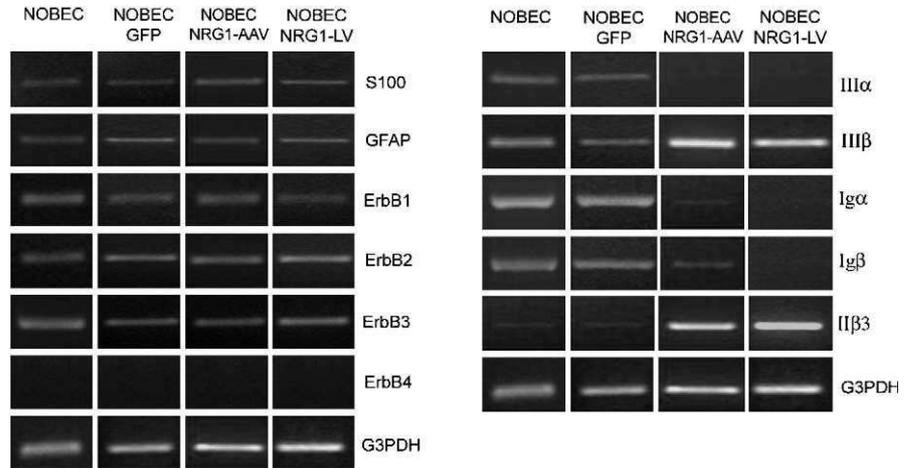


Figure 5

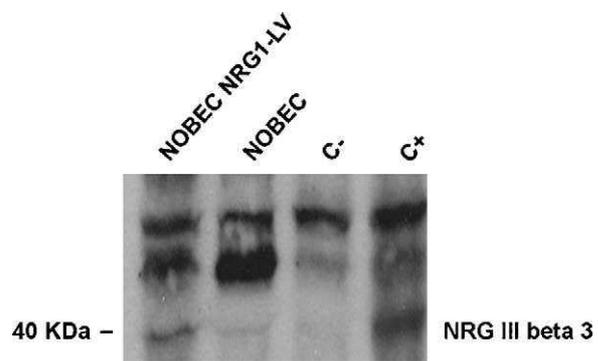
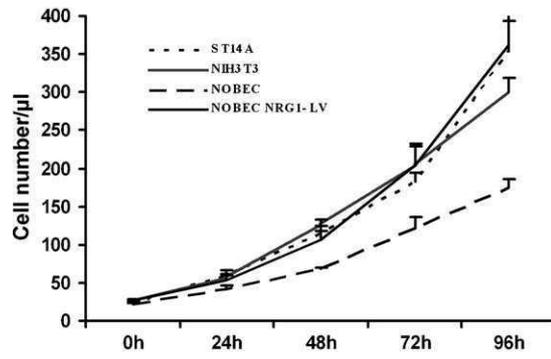
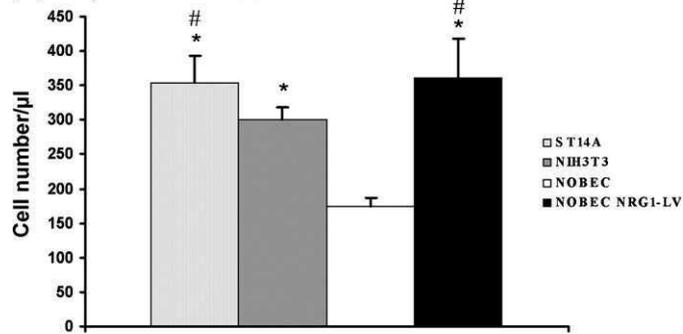


Figure 6

(A) cell proliferation



(B) cell proliferation - 96h



(C) Cell migration

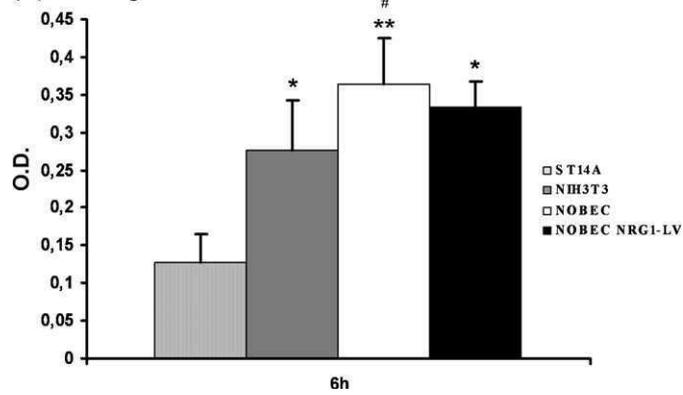


Figure 7

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