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Human olfactory mesenchymal stromal cells co-expressing horizontal basal and ensheathing cells proteins in culture

Modulation of expression of human olfactory mesenchymal stromal receptors

Células estromales mesenquimales de la mucosa olfatoria humana co-expresan proteínas de las células horizontales y de recubrimiento del nervio olfatorio

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Jose E Cardier: designed research, analyzed data and critically read the manuscript.

Reducir a 250 palabras

Introduction: The olfactory neuro-epithelium has an intrinsic capability of renewal during life time provided by the existence of globose and horizontal olfactory precursor cells. Additionally, mesenchymal estromal olfactory cells also support the homeostasis of the olfactory mucosa cell population. Under in vitro culture condition with Dulbecco modified eagle/F12 medium supplemented with 10% fetal bovine serum, tissue biopsies from upper turbinate generated an adherent population of cells which expressed mainly mesenchymal stromal phenotypic markers. Closer examination of these cells has also found co-occurrent expression of olfactory precursors and ensheathing cell phenotypic markers. These results were suggestive of a unique property of olfactory mesenchymal stromal cells as potentially olfactory progenitor cell.

Objective: To ask whether expression of these proteins in mesenchymal stromal cells is modulated upon neuronal differentiation.

Materials and methods: In the current study, we observed the phenotype of olfactory stromal cells under DMEM/F12 plus 10% fetal bovine serum in comparison to cells from spheres induced by serum-free medium plus basic-fibroblast growth factor and epidermal growth factor, commonly inducers of neural progenitors from neurogenic niches.

Results: Expression of mesenchymal stromal ($CD29^+$, $CD73^+$, $CD90^+$, $CD45^-$), horizontal basal (ICAM-1/$CD54^+$, $p63^+$, $p75NGFr^+$), and ensheathing progenitor cells (NESTIN+, GFAP+) proteins was determined in the concurrent cultured population by flow cytometry methodology. Evenmore, determination of Oct 3/4, Sox-2 and Mash-1 transcription factors, as well as neurotrophins BDNF, NT3 and NT4 by RT-PCR in stromal cells was indicative of functional multicellular heterogeneity of the olfactory mucosa tissue sample.

Conclusions: Down regulation of mesenchymal stromal and olfactory precursor proteins in cells from spheres induced by serum-free DMEM/F12 plus growth factors and subsequent differentiation into neurons and astroglial cells was suggestive of a serum-mediated mechanism of mesenchymal stromal protein expression in cells isolated from olfactory mucosa.

Key words: Olfactory mucosa; homeostasis; mesenchymal stem cells.

Reducir a 250 palabras

Introducción. El neuro-epitelio olfatorio tiene una capacidad innata para su renovación durante la vida del individuo gracias a la existencia de las células globosas y horizontales reconocidas precursores olfatorios. De la misma manera, la presencia de las células estromales mesenquimales contribuyen a la homeostasis de la mucosa olfatoria. Cuando una biopsia de mucosa se cultiva en un medio mínimo esencial de Dulbecco modificado suplementado con 10% suero fetal bovino, los explantes generan principalmente una población adherente que expresa marcadores fenotípicos de las células estromales mesenquimales. Un estudio detallado de estas células ha puesto en evidencia la coexpresión de marcadores fenotípicos de precursores olfatorios y de células recubridoras del nervio olfatorio. Estos resultados sugieren una propiedad única de las células estromales mesenquimales como una célula con un posible potencial de progenitora olfatoria.

Objetivo. Observar si la diferenciación celular hacia fenotipos neurales promueve la modulación de la expresión de los marcadores fenotípicos de las celulas mesenquimales.

Materiales y métodos. En este estudio, se caracterizaron las células aisladas en un medio de cultivo suplementado con 10% suero fetal bovino en comparación con mesen-esferas generadas en medio sin suero y suplementado con el factor de crecimiento epidermal y factor de crecimiento de fibroblastos.

Resultados: La determinación por citometría de flujo de los fenotipos de las células cultivadas permitió establecer que la expresión de marcadores de células estromales mesenquimales ($CD29^+$, $CD73^+$, $CD90^+$, $CD45^-$), basales horizontales (ICAM-1/$CD54^+$, $p63^+$, $p75NGFr^+$), y recubridoras del nervio olfatorio (NESTIN+, GFAP+) están presentes en la misma población cultivada. La determinación de los factores de transcripción Oct 3/4, Sox-2 y Mash-1 así como los de las neurotrofinas BDNF, NT3 y NT4 demostró que las células

estromales son funcionales y presentan la heterogeneidad multicelular típica de una muestra de tejido de mucosa olfatoria. La expresión de las proteínas características de las células mesenquimales y precursores olfatorios disminuyó en las células de las mesenesferas inducidas por ausencia de suero en el medio de cultivo.

Conclusión. Las células estromales mesenquimales de la mucosa olfatoria presentar un programa dominante hacia la diferenciación neural.

Palabras clave: mucosa olfatoria; homeostasis; células madre mesenquimatosas.

Seminal work from Schwob et al., (2012) demonstrated that the neuro-epithelium of human olfactory mucosa (HuOM) may be replenished during life time by a single multipotent olfactory progenitor cell, that occur in the basal layer of the olfactory epithelium (1,2). Indeed, it was established that globose basal cells (GBC) are the primary progenitor of the OE playing a role as a main source of sustentacular and olfactory sensory neurons (OSN). Additionally, horizontal basal cells (HBC), the second olfactory progenitor, may take the primary role of progenitor once GBC population is obliterated. Accordingly, renewal of OE occurs as a result of a stringent regulation of cell proliferation and differentiation by both, GBC and HBC olfactory cells (2-7).

Classically, culture of explants from biopsies of human OM has been performed with an enzyme protease pretreatment, which generates a predominant population of mesenchymal stromal cells (MSC) as has been well established by flow cytometry methodology (5,8-10). Subsequent expansion of MSC is performed under in vitro culture condition with fetal bovine serum (FBS) added to the culture medium. As a result of this procedure, OM cells are adherent, with a fibroblast-like morphology and properties such as proliferation and differentiation which are similar to mesenchymal stromal cells from blood bone marrow (10) . Although, same embryological origin may provide similar potential for application in cellular therapy as those from bone marrow, some differences has been reported (10-14). Enhanced capabilities of OM-MSC to differentiate to neural tissue probably occur as a result of its ecto-mesenchymal embryological nature, which has raised a great interest for their possible use in regenerative medicine. Therefore, establishing in vitro properties of olfactory mucosa (OM) tissue biopsies have also proved its efficacy as a source of primary cells for the treatment of neural diseases (3,6,13-18).

There is experimental evidence that neural cells, obtained *in vitro*, from explants of OM may be used for regenerative purpose (11,12,14,19-22). Recent evidences have shown that human OMSC may offered unique properties as a peripheral reporter in some neuropsychiatic disorders (23-27), and chronical diseases such as Alzheimer Disease (28,29), and Parkinson Disease (30).

Taking into a consideration potential of MSC for cell transplantation, several authors have pointed out some issues regarding the uses of FBS for therapeutic applications and research. For instance, due the complex formulation of serum and inconsistency between lots, variability between experimental results has been reported (15,31,32). As a result, it is important to develop better defined media without serum which may modulate metabolic machinery of cells and in some cases expression of characteristic proteins (9).

Being the olfactory mucosa constituted by multiple types of cells, it is likely that ex vivo preparation maintained under culture conditions, may be a source of olfactory progenitors, ensheathing cells and olfactory sensory neurons. Accordingly, establishing appropriate culture conditions of proliferation of mesenchymal stromal, olfactory progenitors and ensheathing cells from tissue explants, and their differentiation in neural cells may offer comprehensive knowledge for cell transplantation.

Here, we asked whether expression of OM-MSC proteins may be modulated by serum-free condition in the culture medium. In doing so, we determined expression of proteins of mesenchymal, olfactory progenitors and ensheathing cells in mesen-neuro-spheres, which take place as a predominant proliferative form under serum-free condition. Neuronal and glial differentiation was preferred under serum-free medium suggesting a neuron-glial oriented differentiation program of olfactory stromal cells.

Materials and methods

Isolation and culture of cells from human olfactory mucosa (HuOM)

All procedures of the study were approved by the Institutional Ethical Committee and it was conducted in compliance with the Declaration of Helsinki for Medical Research Involving Human Subjects. HuOM were isolated from upper turbinate nasal mucosa biopsies collected of patients by endoscopy, as previously described (33). In short: The procedure was performed by an otorrhinolaryngologist under local nasal anesthesia (lidocaine liquid 4% oxymetazoline HCl 0.05%). The endoscopic procedure was practiced using a biting forceps for tissue sampling. Following collection of the tissue biopsy, subjects remained under observation for additional 15 min to ameliorate any additional discomfort. Biopsies of tissue 3 – 6 mm^3 were transported in DMEM/F12 medium with 2% penicillin/streptomycin and kept between 4 to 8 ºC until dissection. Tissue sample was further dissected in two fragments: one of the two fragments was fixed in 4% p-formaldehyde for histological studies using regular H&E histochemistry and immunostaining. The second fragment was used for generating tissue explants of 0.5 - 1 mm^2 using micro scissors under sterile conditions; 1 – 3 explants/well were placed in 24 well plates with DMEM/F12 culture medium supplemented with 10% FBS, 0.5% penicillin/streptomycin and glutamine (DMEM/F12-CM) in a CO_2 incubator.

Cell culture reagents

Dulbecco Modified Eagle Medium (DMEM/F12) culture medium was from GIBCO (NY, USA), fetal bovine serum (FBS) was from SIGMA (St. Louis, MO, USA); TrypLE Express (GIBCO), N2 supplement (GIBCO), recombinant human basic-fibroblast growth factor (b-FGF, R&D systems, 234FSE), recombinant human epidermal growth factor (EGF, GIBCO, PHG0311),

Forskolin (F6886) and Trizol reagent was obtained from SIGMA (St. Louis, MO, USA). Antibodies for immunofluorescence: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies anti-human: CD29, CD54, CD73, CD90, NESTIN, and CD45 were purchased from Becton Dickinson (San Diego, CA, USA); CD271-FITC was from Biolegend (Minneapolis, MN, USA), GFAP from DAKO, and tubuline β-III was from Millipore (table 1).

Osteogenic, chondrogenic and adipogenic differentiation of Hu OM-MSC

The multipotential capacity of OM-MSC was examined by culturing cells in osteogenic, chondrogenic and adipogenic differentiation media, as previously described (17,34). Briefly, OM-MSC from passages 2 to 5 cultured in DMEM/F12-CM medium were harvested using TrypLE express solution and seeded at 250000 cells/cm^2 in 24 wells plate in osteogenic, chondrogenic and adipogenetic differentiation medium. Under each condition cells were kept for 21-28 days replenishing the medium every 4-5 days. Once reached end point of differentiation, cell culture was washed with PBS and fixed in 10% p-formaldehyde. Alizarin red, alcian blue and oil red histochemical stainings were performed to reveal calcium deposits for osteogenesis, glucosamino glucan for chondrogenesis and cytoplasmic fat drops as indicator of adipogenesis, respectively. Bright field photomicrographies were acquired with a Zeiss Axiovert Observer D1 inverted microscope equipped with a Tucsen ICC 5.0 ICE digital camera routed to a computer.

Flow cytometry analysis of OM stromal cells (OM-MSC)

Adherent cells generated from explants began to proliferate and migrate from the tissue. A crown of cells surrounding explants becomes confluent after 7 to 10 days in culture. Cells were collected and transferred to plates with a larger growing area until passage 2 to 5 for analysis. Phenotypic characterization of cells was achieved by flow cytometry methodology

for the expression of extracellular MSC (CD29, CD45, CD73, CD90) proteins and intacellular ensheathing/neural progenitor (NESTIN, glial fibrillary acidic protein (GFAP, DAKO), HBC (ICAM-1/CD54, p63 (DAKO), and low affinity neural growth factor (FITC-NGFr/p75, Biolegend) proteins. Simultaneous negative control staining reactions were performed by incubating cell preparation with the corresponding IgG isotype FITC or PE derivative. In case of non conjugated primary antibody, cell preparation was analyzed side by side using no primary but secondary as negative control.

Intracellular labelling was performed following Cytofix/Cytoperm BD (554722) kit from Becton and Dickinson datasheet instructions, in short: 350 ul of Cytofix were applied to 10^6 cells and kept on ice for 20 min. Then 450 µl of Cytoperm (1/10 dilution with water) and cell suspension centrifuged and resuspended in cytoperm in a quantity of volume enough for all determinations. Data collection and analysis of the fluorescent intensities were made using a FACS Calibur (Becton Dickinson, San Jose, CA). Ten thousand events were acquired and analyzed using the Cell Quest software program.

Detection of transcription factors and neurotrophins indicatives of olfactory progenitors and ensheathing/neural progenitor stromal cells by RT-PCR

Total RNA was extracted from 90% confluent OM-MSC in DMEM/F12-CM using Trizol according to the manufacturer's instructions. Reverse transcription was carried out using random hexamer oligonucleotides and 4 U AMV reverse transcriptase (Promega, Madison, WI) for cDNA synthesis. RNA quality was assessed by Agilent 21000 Bioanalyzer (Foster City, CA) and quantified by Nanodrop (Wilmington, DE). PCR amplification of the cDNA was then performed using Platinum TaqDNA polymerase (Invitrogen) and specific oligonucleotides (table 2) for the detection of: transcriptional factors Oct 3/4, Sox-2, *Mash*-1 and neurotrophins, brain derived nerve factor (BDNF); neurotrophin 3 (NT3) and neurotrophin 4 (NT4); and ß-

actin transcripts from 40ng of RNA equivalent cDNA, with a blank RT control. PCR conditions were a denaturing step for 2 min at 94ºC, followed by 35 cycles of 1 min at 94ºC; annealing for 1 min at 50ºC (Mash-1), 52ºC (Oct 3/4, NT3 and NT4), and 54ºC (Sox-2 and BDNF); and extension for 1 min at 72ºC. Analysis of the PCR products was performed by comparing them with the predicted PCR fragment size after ethidium bromide staining of the PCR products separated by electrophoresis in a 1.8% agarose gel.

Adaptive response of olfactory stromal cells to culture medium condition

All experiments under serum-free condition were performed with cells from passage 3 to 5 grown in DMEM/F12-CM culture condition. Once cells become confluent, the culture medium was replaced with a DMEM/F12 serum-free medium. Then, after five days, cells were collected by TrypLE Express solution treatment and transferred into a serum-free DMEM-F12 culture medium containing N2 supplement with Hu-EGF (20 ng/ml) and b-FGF (20 ng/ml) (DMEM/F12-GF) added to the medium.

Under DMEM/F12-GF culture condition, mesen-neuro-spheres occur after 2 days and proliferate as spheres or no to low adherent stroma. After 7 days mesen-neuro-sphere were harvested, dissociated after incubation with TrypLE Express solution, characterized by flow cytometry. Functional properties were studied under specific conditions, as described below.

Studying cells from mesen-neuro-spheres-induced by serum-free DMEM-GF

Flow cytometry analysis: Mesen-neuro-spheres-induced by DMEM-GF culture condition were collected and dissociated to single cells after incubation with TrypLE Express solution by 15 min. Cells were labelled directly with the conjugated primary antibody (3 µl for 10^5 cells, plasma membrane immune labelling) or fixed and permeabilized with a cytofix/cytoperm permeabilization kit. Then, cells were incubated by 30 min with conjugated primary antibodies or the corresponding isotype. After incubation with the antibody, each tube was rinsed with

PBS and centrifuged at 1500 rpm for 5 min. In each experiment percentage of immunopositive cells was determined using the corresponding isotype or the secondary antibody with no primary as negative control.

Neurogenic differentiation of cells from mesen-neuro-spheres-induced by serum-free DMEM-GF culture condition

Spheres obtained under DMEM/F12-GF condition were examined in their neurogenic potential by culturing single cells in neural differentiation media. Briefly, single cells were seeded at 3.5 $x10^4$ cells/cm^2 in 24 wells plate on coated coverslips with poly-L-lysine (10 µg/ml) solution. Our protocol for elevating intracellular cAMP was modified from Deng et al 2006. In addition to the primary culture condition to generate mesen-neuro-spheres, single cells were differentiated in serum-free DMEM/F12 plus N2, α-trans retinoic acid (1 µM), R-2625, SIGMA) and forskolin (5 µM, F6886, SIGMA) by 7 days after plating (DMEM/F12-RA+FORSK). Forskolin, a phosphodiesterase inhibitor increase cAMP levels and retinoic acid induce neural differentiation. DMEM-GF medium was changed at day 4 with freshly prepared DMEM-GF medium. At day 7, the cell culture medium was removed, and cells fixed with p-formaldehyde 4% in PB 0,1 M by 15 min, once fixed plates were maintained in PBS solution at 4 ºC until immunostaining was performed.

Immunostaining of dissociated mesen-neuro-sphere growth on coverslips

Cells on coverslips were permeabilized with PBS (0.01 M) containing 0.1% Triton X-100 at RT for 30 minutes. Nonspecific sites were blocked with 2% normal goat serum (Invitrogen) for 30 minutes. Cells were then incubated with the primary antibody TUJ-1 (1/200 Mouse monoclonal tubulin β-III antibody, Millipore) and GFAP (1/500 rabbit GFAP from DAKO) at 4°C overnight. Fluorescent secondary antibodies (Vector Labs) at a dilution of 1:200 were applied for 1 hour at RT and immunoreactivity detected under fluorescence using DAPI as

counterstaining. Coverslips from control and experimental conditions were stained side by side in a single and double labeling paradigm using the same batch of antibodies.

Counts of TUJ-1 and GFAP positive cells were determined by single or double labelling for glial and neuron cells in at least 3 coverslips for condition from 3 independent experiments.

All histological and immunohistochemical images were acquired from a Zeiss Axioplan 2 D1 microscope equipped with a Tucsen 5.0 ICE digital camera (China).

Statistical analysis

Results from flow cytometry analysis are reported as median ± 25 percentile of the median from at least 9 independent experiments. We tested whether median of the variable is affected by DMEM-GF with respect to DMEM-CM experiments for statistical significance using *U* of Mann Whitney non parametric analysis. A value of *P* less than 0.05 was considered significant.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Results

Culturing cells from explants of HuOM

A typical cytoarchitecture of pseudostratified epithelium lying on a basal lamina was observed from tissue sections under H&E staining protocol (figure 1A). Bowman secretory components of the *lamina propia* occurred underneath the basal lamina (figure 1A). Detection of expression of β-III tubulin protein by immunofluorescence protocols was suggestive of immature neurons immunolabelled with TUJ-1 antibody (figure 1B). This observation

confirmed that the biopsy of the tissue sample was collected from the sensory epithelium of the olfactory nasal cavity.

Explants from HuOM biopsies were seeded on cell culture plates with regular medium (DMEM/F12-CM). After 48 h, cells migrated from explants, and generated a stroma that reached confluence after 10 to 15 days. A predominant population of fibroblast-like cells was observed migrating from explants (figure 1C). In subsequent experiments we studied phenotypic markers and differentiation potential of these stromal cells under (DMEM/F12-CM) culture condition. Subsequently, we studied adaptive response of stromal cells under free serum medium and found that stromal cells proliferated as mesen-neuro-spheres (figure 1D). Then, we identified their potential of differentiation to neural cells under conditions provided by culture medium supplemented with growth factors (DMEM/F12-GF).

Description of HuOM-adherent cells by flow cytometry methodology

Stromal cells from HuOM tissue explants were cultured with regular medium (DMEM/F12-CM). We determined expression of CD29, CD73 and CD90 proteins, well established markers of MSC by flow cytometry technique (figure 2 A-C). Furthermore, in the same population of cells we also determined expression of CD54 (figure 2D) and p63 (figure 2E), which was suggestive of expression of horizontal-like basal cells phenotypic markers. Besides, in this cultured population we also determined NESTIN (figure 2F) and $GFAP^{+}$ (not shown) immunopositive cells, which are proteins commonly expressed in ensheathing/neural-like progenitors. Altogether, these results were suggestive of a mesenchymal stromal cell population of cells that co-expressed proteins from olfactory precursors and neural progenitor cells. Interesting, less than 7% of cells were positive for CD271 (p75NGFr) and cytokeratin 5. Noticed that the highest percentage of cells determined by flow cytometry analysis is corresponding with expression of MSC proteins; as a consequence, findings of horizontal-like

basal and ensheathing cells protein markers was suggestive of concurrent expression of these markers in the MSCs population.

Coherent with these findings, we established by PCR methodology expression of transcription factors (Oct 3/4, *mash*-1 and Sox 2) and neurotrophins (BDNF, NT3 and NT4), commonly associated with sustentacular, HBC, GBC and ensheathing/neural-like progenitor cells, in the adherent population from HuOM tissue biopsies (figure 3A). Altogether, these results confirmed a spontaneous co-expression of olfactory and neural progenitor protein markers in the MSC cultured population. In agreement with data from flow cytometry studies, further functional assays demonstrated that adherent MSC may differentiate, under well established specific culture conditions, into osteoblasts, chondroblasts, and adipocytes respectively (figure 3B-D).

Growing of stromal cells in spheres-induced by serum-free DMEM/F12 GF culture medium

We investigated the capacity of MSC from HuOM to generate mesen-neuro-spheres. For this purpose, confluent monolayers of MSC under DMEM/F12-CM were adapted to serum free DMEM/F12 condition for 5 days. Then, cells were harvested and cultured in serum-free DMEM/F12 medium supplemented with growth factors (DMEM/F12-GF). Under these culture conditions, cells showed noticeable phenotypic changes without decreasing cell viability (figure 1D). After 3 - 4 days, cells grew forming spheres, which were rather similar to neurospheres from neural progenitor cells. The same pattern of cell growth was maintained through several passages in DMEM/F12-GF.

Descriptive phenotypical characterization of mesen-neuro-spheres obtained from HuOM-MSC cultured in DMEM/F12-GF

Mesen-neuro-spheres induced by serum-free adapted stromal cells (at least 9 subjects) under DMEM-GF culture condition were harvested, dissociated and analyzed by flow cytometry protocol. Under DMEM/F12-CM (10% FBS) culture condition, the median of percentage of cells expressing mesenchymal stromal proteins (CD29, CD73 and CD90) was around 98% with a low variability. In contrast, we observed reduction of expression of MSC proteins (figure 4 A, C, E; $P < 0,0001$; table 3). Otherwise, HBC protein marker ICAM-1/CD54 protein was highly variable; eventhough, we found a significant reduction in the median of percentage of cells expressing the (figure 4B; $P< 0,05$; table 3).

Noticeable, the percentage of $NESTIN^+$ and $GFAP^+$ cells was not modified under this condition (figure 4 D and F). Overall protein expression of HBC-like and ensheating cell-like proteins was highly variable in the cultured population under DMEM/F12-CM and DMEM/F12-GF. However, altogether, these findings were suggestive of modulation of expression of MSC and HBC-like proteins metabolically induced by serum-free DMEM-GF culture condition with preservation of NESTIN and GFAP proteins suggesting stability of the neural progenitor phenotype.

Differentiation of cells from mesen-neuro-spheres from HuOM-MSC induced by DMEM-GF

We examined the capacity of cells from spheres-induced under DMEM/F12-GF to differentiate into early neurons (TUJ-1). For this purpose, single cells from spheres were seeded on PDL-coated coverslips and cultured under DMEM/F12-GF (figure 5A, C) or DMEM/F12 RA+FOSK) for 7 days (figure 5B, D). After seeding, single cells become attached to the substrate in both culture conditions and either generate spheres attached to the PDL

(figure 5A, arrow) or a monolayer with medium to high complexity (figure 5B)**.** We determined a strong expression of TUJ-1 in both culture conditions (figure 5C, D). Interesting, in both experimental conditions expression of TUJ-1 was found either in mesen-neuro-spheres (figure 5C, arrows) or in single cells (figure 5D). We found no differences between cell numbers of TUJ-1 immunopositive cells in both experimental conditions (results not shown). Then, we asked whether single cells grown under DMEM/F12-GF and DMEM/F12 RA + FORSK would differentiate in neuron or glial cells. To address this question, the expression of TUJ-1 and GFAP proteins was examined after cells were plated and cultured by 7 days in culture mediums as quoted above. We determined TUJ-1 expression in cells under both conditions. However, whereas GFAP expression was colocalized with comparable expression of TUJ 1 under DMEM/F12-GF (figure 6A, C, E); unexpectedly, under DMEM/F12 RA+ FORSK the expression of GFAP was restricted to the nuclear area (figure 6B, D, F). Findings as we obtained from these experiments are suggestive of two reservoirs of GFAP protein in the MSC population. As a consequence, there was not colocalization of both immunomarkers; however, counts of number of cells double labeled were not different under these culture conditions (not shown). These results are suggestive that either DMEM/F12-GF or DMEM/F12 RA+FORSK may induce differentiation to TUJ-1 immunopositive cells from mesen-neurospheres. However, ectopic expression of GFAP was promoted by DMEM/F12 RA+FORSK.

Discussion

In this study, we have established culture conditions for biopsies of HuOM without pretreatment with enzyme proteases. First at all, we observed a predominant monolayer of stromal cells under DMEM/F12-CM culture condition, whereas after serum-free adaptive protocol, mesen-neuro-spheres induced by DMEM/F12-GF culture condition were isolated

from the cell population. After proliferation and expansion of stromal cells, they were further identified as MSC by flow cytometer analysis according to guidelines of the ISSCR organization (13).

For our knowledge, this is the first demonstration of MSC from human OM spontaneously expressed antigens of olfactory basal progenitors and ensheathing/neural-like progenitor cells. Previous results have also shown that mice MSC from blood bone marrow also expressed nestin (100%), eventhough βIII-tubulin and neurofilaments, expressed than 15% altogether (8). In contrast, we determined more than 80% of cells immunopositive for NESTIN, around 90% of P63 and 50% of ICAM-1/CD54 and 40% of GFAP. These findings suggested that HuOM-MSC indeed have a pro neural-like basal condition in the concurrent population associated with typical phenotypes found in the OM, i.e.g., HBC, GBC, sustentacular and ensheating cells.

Our results are suggestive of ex vivo explants from OM contain stromal cell components expressing phenotypes of the primary tissue, e.g., olfactory precursors, ensheathing/neural-like progenitor and mesenchymal stromal cells of the olfactory niche. In addition, these findings also suggest that richness of stromal cell phenotypic markers determined in this culture condition is indicative of complete functional explants preparation of human olfactory epithelium.

Following our tissue collection protocol, samples examined had a characteristic well structured cyto-architecture of OE as shown by H&E staining. Evenmore, expression of TUJ-1 antigen in the tissue sample is indicative of immature neurons in the olfactory neuro-epithelium. These findings confirm that tissue samples under study were collected from upper turbinates where constitutive neuronal maturation and turnover is actively contributing to the morphology and complexity of the olfactory sample. Additionally, these results are in

agreement with the detailed cellular characterization of autopsied human olfactory mucosa samples recently reported (1,2,7,11).

Culturing explants under DMEM/F12-CM condition, proliferating cells migrated from explants and a stromal monolayer of cells expressed $CD29^{+}$, $CD73^{+}$, $CD90^{+}$, $CD271^{+-}$, and was negative for $CD45^{-}$ antigen; altogether established markers of MSC. A reduced population of CD271 (p75, NGFr) and cytokeratin 5, was also determined. Noticeable, findings of ICAM-1/$CD54^{+}$ (5,35) and $p63^{+}$ (6, 7), as well as $NESTIN^{+}$ and $GFAP^{+}$ immunopositive cells detected in the growing population are indicative of the plethora of proteins expressed in the olfactory stromal characteristic cells of the mucosa, e.g. GBC, HBC and ensheathing/neural-like cells.

Based on these results shown above, HuO-MSC expressed mRNA messages for Oct 3/4, Sox-2 and *mash*-1 transcription factors that has been associated to olfactory basal cells which participate in the renewal of olfactory epithelium and bulb interneuron specification, migration and differentiation. In addition, mRNA messages were found for growth factors BDNF, NT3 and NT4. For instance, expression of neurotrophins in the cultured population is suggestive of survival signaling typical for ensheathing/neural-like progenitor cells in the OM tissue sample. In previous studies, spontaneous neural transdifferentiation has been suggested for bone marrow (BM) MS cells from rodents (8,36) and humans (10). For instance, MS spontaneously expressed NESTIN under serum-free condition. In contrast, a MS profile as we shown in this study was described for cultured cells isolated after enzymatic digestion and dissection from the *lamina propria* (10,31). Eventhough, expression of ICAM-1/$CD54^{+}$ immunopositive cells was also determined in the cultured sample, suggesting that HBC antigen may be coexpressed in the BM cultured population, these findings were not discussed (10).

Evenmore, CD105 plasma membrane marker, a well accepted MS marker seems not to be strongly expressed in OM MSC, as we also have determined (37).

In this study, HBC-like antigen has been identified using well characterized antibodies tested for the in vivo expression of CD54+/ICAM-1 and p63, a transcription factor as established by previous reports (5-7, 20). Our findings of ICAM-1/CD54^{+} and p63^{+} transcription factor immunopositive cells in our experimental paradigm are the first time demonstration of in vitro proliferation of human mesenchymal stromal cells that spontaneously expressed antigens of HBC and ensheathing cells. Seminal work by Murrell et al. 2005 demonstrated multipotent stem-like cells in the *lamina propria* and olfactory epithelium. Eventhough a detailed and extensive analysis was perfomed in order to establish the source and origin of stem cells; results from these authors suggested that neural-like stem cells occurred in both neuroepithelium and *lamina propria,* thus, findings from those studies were not conclusive. In support of these findings, we determined high percentage of MS, OP and ensheathing/neural like phenotypic markers. Expression of NESTIN^{+} cells in stromal and spheres under DMEM/F12-CM and -GF and our results support the neurogenic potential of these cells.

Eventhough, the multipotency of MS cells has been clearly established for decades, not all MS cells isolated from different tissues are equi-functional (12). For instance, previous reports have showed that OM stromal cells isolated from *lamina propria* have reduced potential to differentiation in mesodermal tissues (10). In contrast, olfactory mucosa mesenchymal stromal cells in this study under in vitro specific culture conditions differentiated in osteoblasts, chondrocytes and adipocytes, as a consequence these findings support the multipotency already described for MS from OM cells.

Because neural stem cells classically proliferate and self-renew as neurospheres, and it has been shown for previous reports that the lack of serum stimulate growing of stromal cells like

spheres, we adapted stromal cells to this condition (38,39). We perfomed and adaptive experiment where cells remained in DMEM/F12-CM 10% FBS until confluency, then cells were kept in DMEM/F12-0% FBS. Finally, stromal cells were collected and passaged to DMEM/F12-GF to promote proliferation as neurospheres-like spheres. Under this condition clonal spheres appeared after 3 to 5 DIV.

As a result of the DMEM/F12 GF culture condition, the expression of MS phenotypic markers was reduced, whereas the expression of both NESTIN and GFAP was not affected with respect to DMEM/F12-CM. Importantly, eventhough NESTIN antigen seems ubiquitous and not a specific marker of neural stem cells, because it is also transiently expressed in other cell progenitors as muscle, human bone marrow mesenchymal stromal cells and some epithelial derivatives, it has classically recognized associated to neural progenitors in neurogenic niches of the central nervous system. High expression of NESTIN and GFAP in human olfactory mucosa at low passages, as we have determined support the presence of constitutive ectodermal neurogenic-like progenitors in the stromal cell population from the olfactory sample.

Our findings are the first evidence of highly expressed $NESTIN^{+}$ cells under DMEM/F12-CM and in DMEM/F12-GF culture condition. In contrast, other reports have showed a medium to low expression of NESTIN as well as no expression of GFAP positive cells (32). In our experimental model we have obtained proliferation of stromal cells and spheres as well as expression of a phenotype of ensheathing/neural-like progenitor induced by the DMEM/F12-CM or -GF medium.

Differentiation of MSC in immature neurons TUJ-1+ from HuOM mesen-neuro-spheres in DMEM/F12-GF supported the importance of this experimental approach in therapeutics and possible applications in regenerative medicine.

In this work we have shown that HuOM-MSC spontaneously proteins associated with olfactory precursors such as HBC and GBC and ensheating cells. The expression of neural markers has been reported on MSC from other sources (8,40). It is possible that cells co-expressing MSC and neural progenitors may represent a special population with a specific pattern for neural differentiation.

In summary, our work demonstrated mesenchymal stromal cells displaying phenotypes of olfactory progenitors and ensheathing cells, which down modulated membrane receptors in DMEM-F12/GF medium. We have also have shown that human OM contains a stromal mesenchymal population responsive to lack of serum which differentiate to neurons and glial cells. These results support the principle of differentiation of stromal mesenchymal cells to neurons which can be used in research and potentially for regenerative therapies in clinic.

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Conflict of interest

Authors declare that they do not have any conflict of interest to disclose.

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Figure 1. Biopsy specimens from HuOM provided sections for histology studies and explants which generated stromal cells and mesen-neural-spheres under specific culture conditions. A) tissue section (5 µm) stained with H&E protocol showed a typical architecture of OE from sensory mucosa: a) epithelium; b) basal lamina; c) Bowman ducts; d) olfactory parenchyma. B) Neuronal β-III tubulin (TUJ-1, green) immunofluorescence counterstained with DAPI (blue) demonstrated that biopsies tissue samples were collected from sensory mucosa. In the figure :a) epithelium; b) TUJ-1 (green) immunoabelled cells; c) bowman duct C) Tissue explants generated elongated fibroblast-like cells under DMEM/F12-CM culture condition. D) Mesen-Neuro-spheres were generated from stromal cells after adaptive culture protocol under serum free DMEM/F12-GF culture condition. Scale bar: 100 um (A, B, C, D).

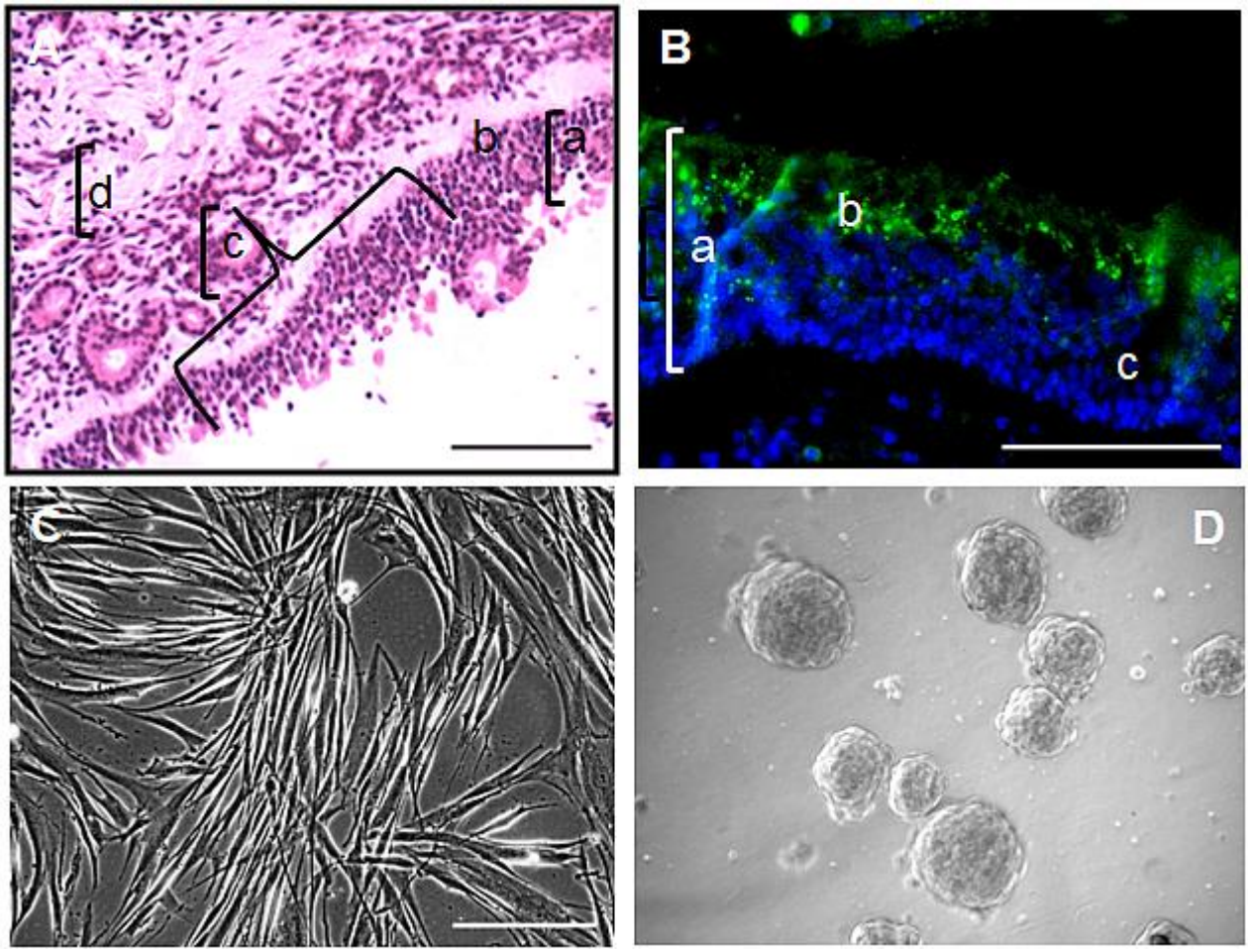


Figure 2. Stromal cells from OE tissue explants proliferated under DMEM/F12-CM. A - C) flow cytometry histograms: Percentage of immunopositive CD29, CD73 and CD90 cells was indicative of mesenchymal stromal cells. Negative controls were labelled with the correspondent IgG isotype (black histograms). Dot blots (D – F) from stromal cells were negative for CD45 (not shown), slightly positive for CD271 and CitK5. Percentage of immunopositive CD54/ICAM-1, p63, and NESTIN cells was indicative of horizontal (D and E) and ensheathing (F) antigens spontaneously expressed in mesenchymal stromal cells. All histograms are representative of one individual in a single analysis.

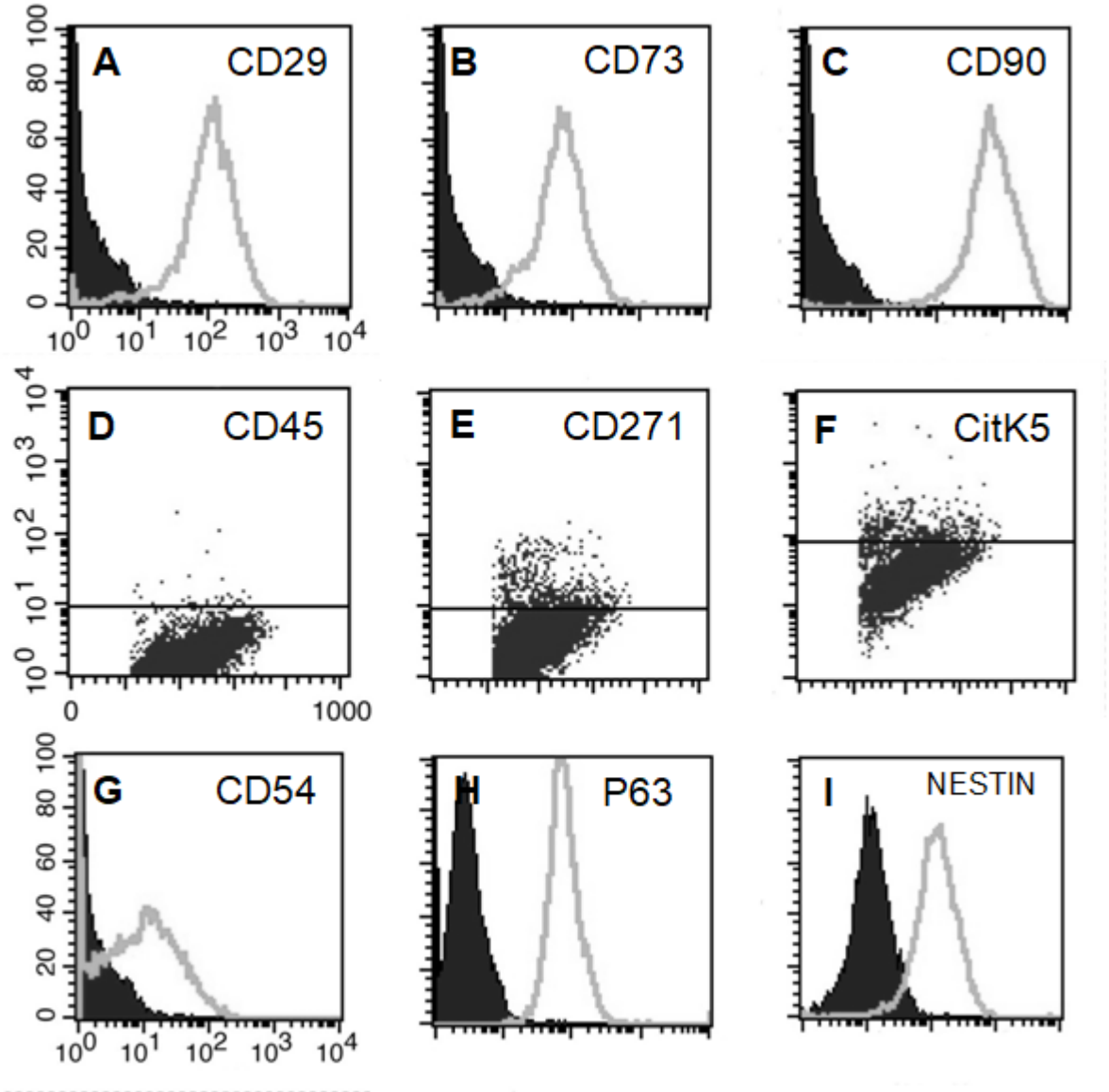
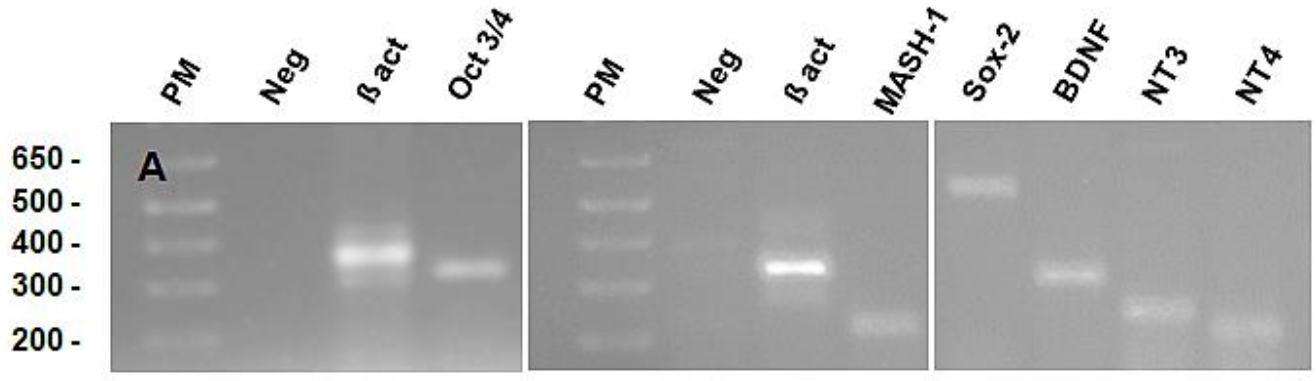


Figure 3. Stromal cells expressed transcription factors and neurotrophins and differentiated in characteristic mesodermal derivatives. RT-PCR from stromal cells was performed as described in the methods section using specific primers for Oct 3/4, Sox-2, Mash-1, BDNF, NT3 and NT4 (Table 2). A) OM stromal cells constitutively express early transcription factors Oct 3/4, Mash-1, Sox-2 (HBC and GBC) olfactory precursors, growth factors BDNF, NT3 and NT4 (ensheathing cells). Results are representative of at least three independent experiments. (B) Stromal cell differentiated after 14-21 days into mesodermal lineages, a) mesenchymal stromal cells in DMEM/F12-CM. b) calcium deposition in red suggesting osteoblasts, c) adipocytes (white head arrows) and d) alcian blue deposition in chondrocytes . Micrographs show calcium deposits (red deposits in b), oil red fatty acid inclusions (c) and Alcian blue positive stain (d) in stromal cells. Results are representative at least of 3 independent experiments.



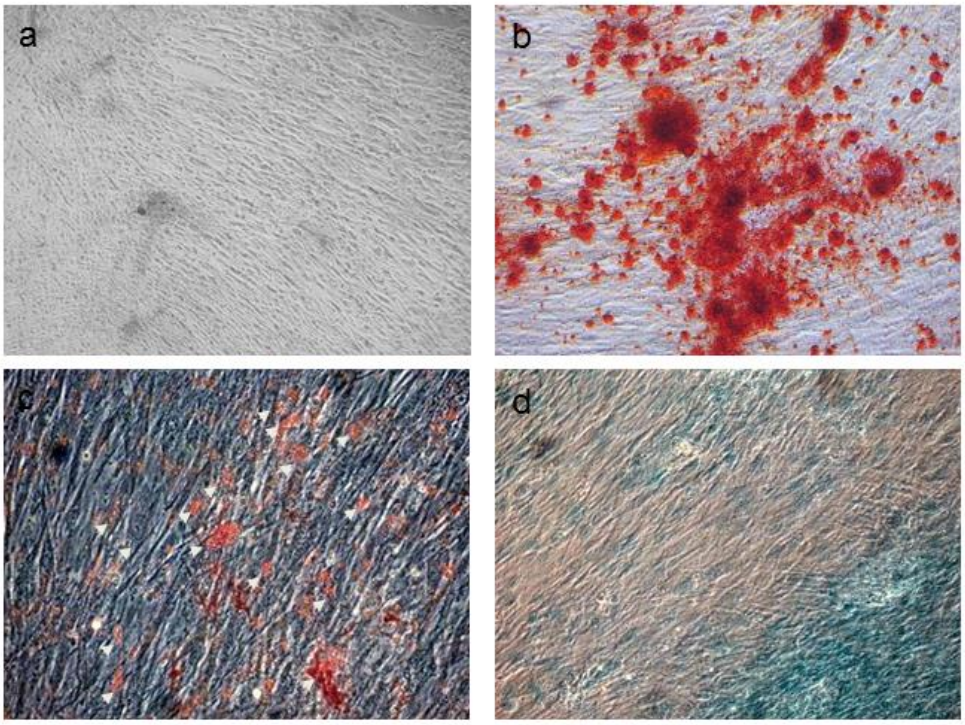
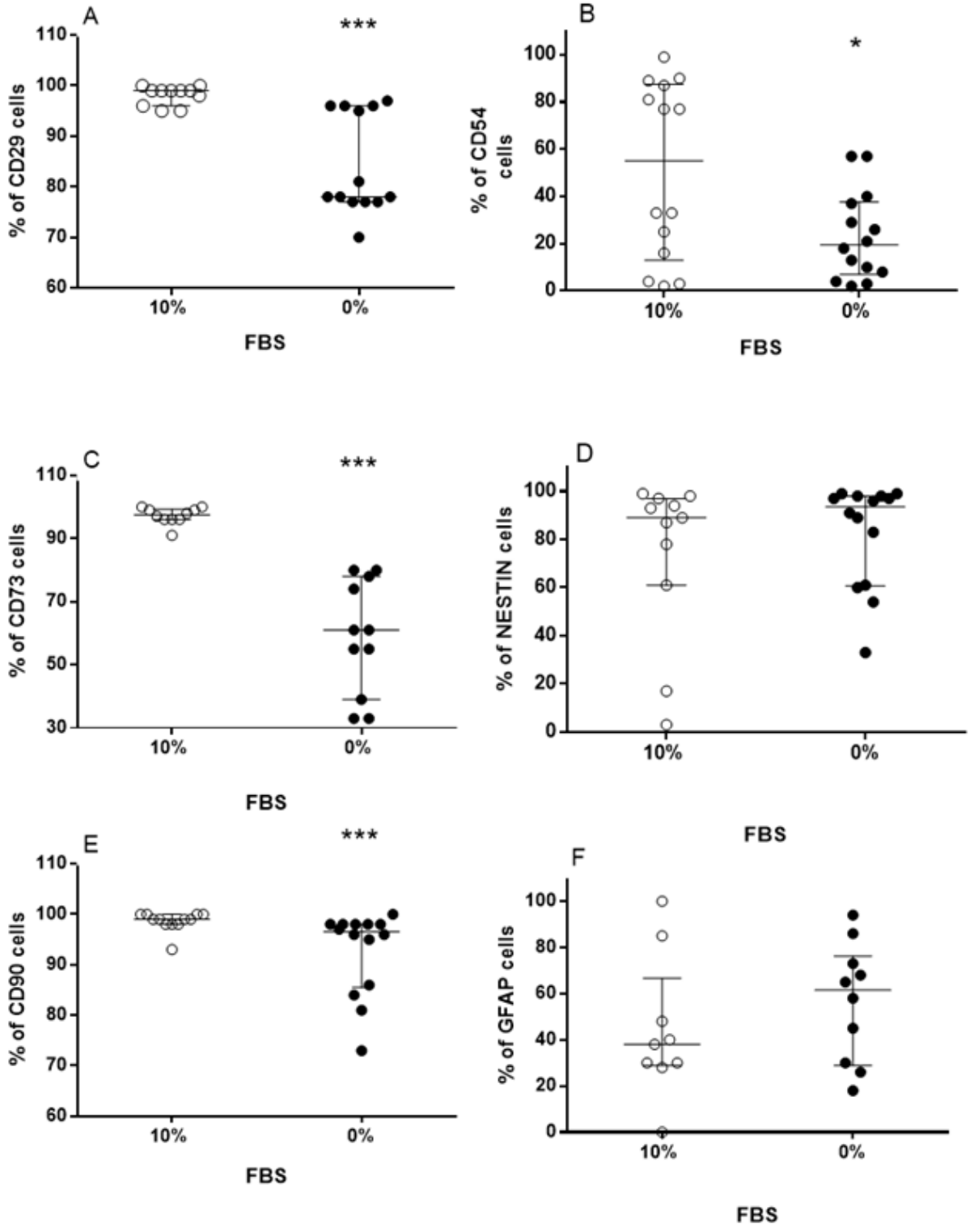


Figure 4. Mesen- neuro- spheres-induced by serum free DMEMF12-GF culture condition expressed lower antigens for mesenchymal and HBC olfactory precursor cells. Cultured HuOMSC adapted to serum free condition were grown under DMEM/F12-GF. Stromal cells-induced spheres were harvested and percentage of CD29, CD73, CD90; ICAM-1/CD54 cells was determined. Overall, mesenchymal and HBC markers were reduced. Graphs are the percentage of A) CD29, C) CD73, E) CD90 and B) ICAM-1/CD54, D) NESTIN, F) GFAP cells determined under DMEM/F12-CM with respect to DMEM/F12-GF. Results are the median of at least 9 to 14 subjects. Each determination was performed in single side by side DMEM/F12-CM and DMEM/F12-GF cell sample from each subject. *** $P < 0.0001$; * $P < 0.05$.


A
***
% of CD29 cells
110
100
90
80
70
60
10%
0%
FBS
B
*
% of CD54 cells
100
80
60
40
20
0
10%
0%
FBS
C
***
% of CD73 cells
110
90
70
50
30
10%
0%
FBS
D
% of NESTIN cells
100
80
60
40
20
0
10%
0%
FBS
E
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% of CD90 cells
110
100
90
80
70
60
10%
0%
FBS
F
% of GFAP cells
100
80
60
40
20
0
10%
0%
FBS

Figure 5. HuOM-MSC mesen- neuro- spheres expressed TUJ-1 protein of immature neurons. Differentiation of mesen-, neuro- spheres from MSC grown in coverslips in DMEM/F12-GF and DMEM/F12-RA + FORSK. MSC were adapted to the lack of serum and seeded in DMEM/F12-G. Single cells from spheres were cultured in DMEM/F12-GF (A, C) and in DMEM/F12-RA+FORSK (B, D). Cells differentiate as mixed stroma and spheres (A, white arrow) or a predominant stroma (B). TUJ-1 (green) immunolabelling was found in spheres (C) and in single cells (D) from olfactory MSC grown in coverslips in DMEM/F12-GF and DMEM/F12-RA + FORSK. Counterstaining with DAPI. Scale bar: A, B (100 µm); C (50 µm); D (25 µm).

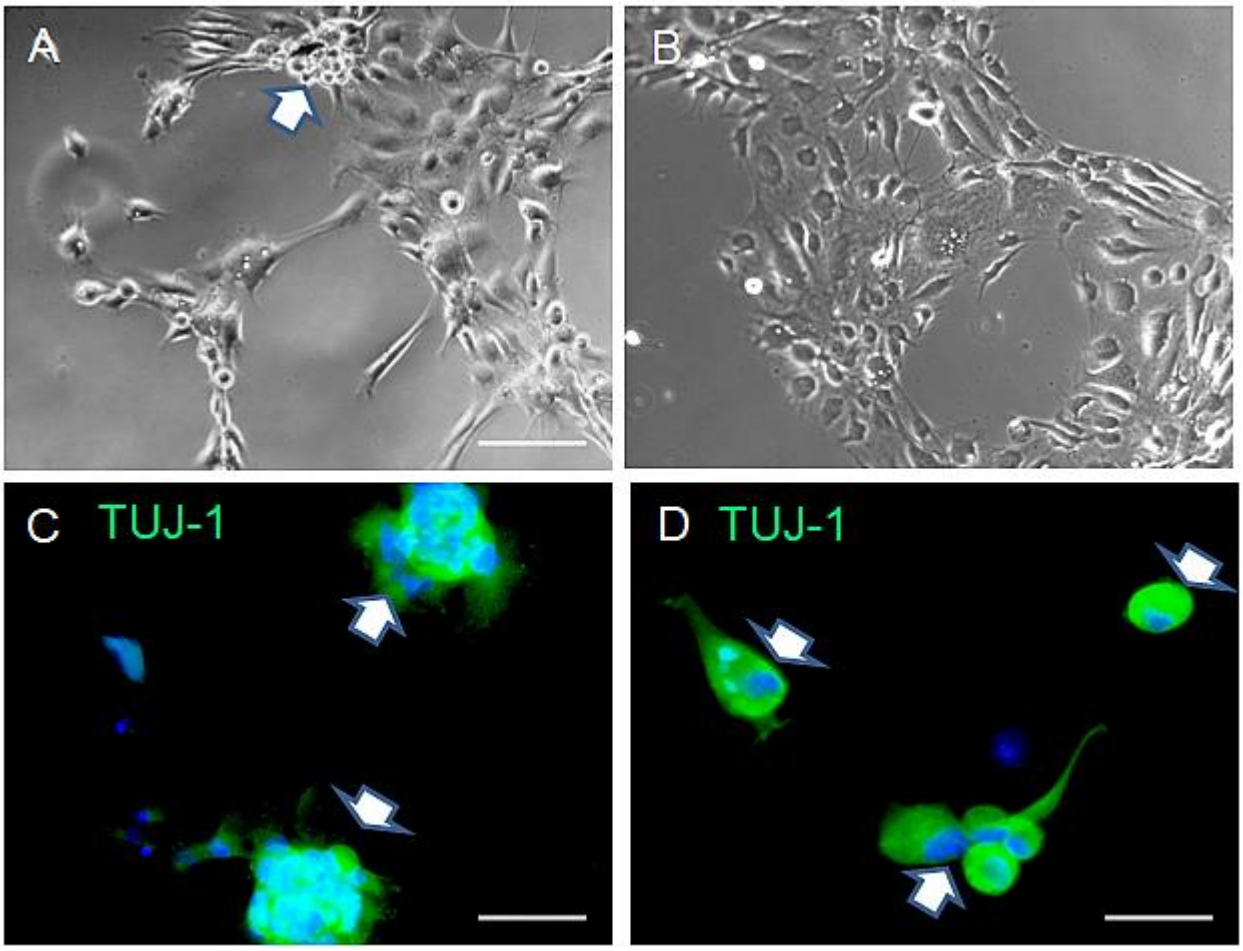

Figure 6. HuOM-MSC expressed protein for neurons and glial cells.
MSC were adapted to the lack of serum and seeded in DMEM/F12-GF until spheres appeared and become confluent. Single cells from spheres were cultured in DMEM/F12-GF (A, C, E) and in DMEM/F12-RA+FORSK (B, D, F). TUJ-1 immunopositive cells were detected in stromal cells in DMEM/F12-GF (A, C, E) and in DMEM/F12-RA+ FORSK (B, E, F). Coexpression of TUJ-1 and GFAP was no different in DMEM/F12-GF (E) and in DMEM/F12-RA+FORSK (F). Scale bar: 100 µm (A, B).

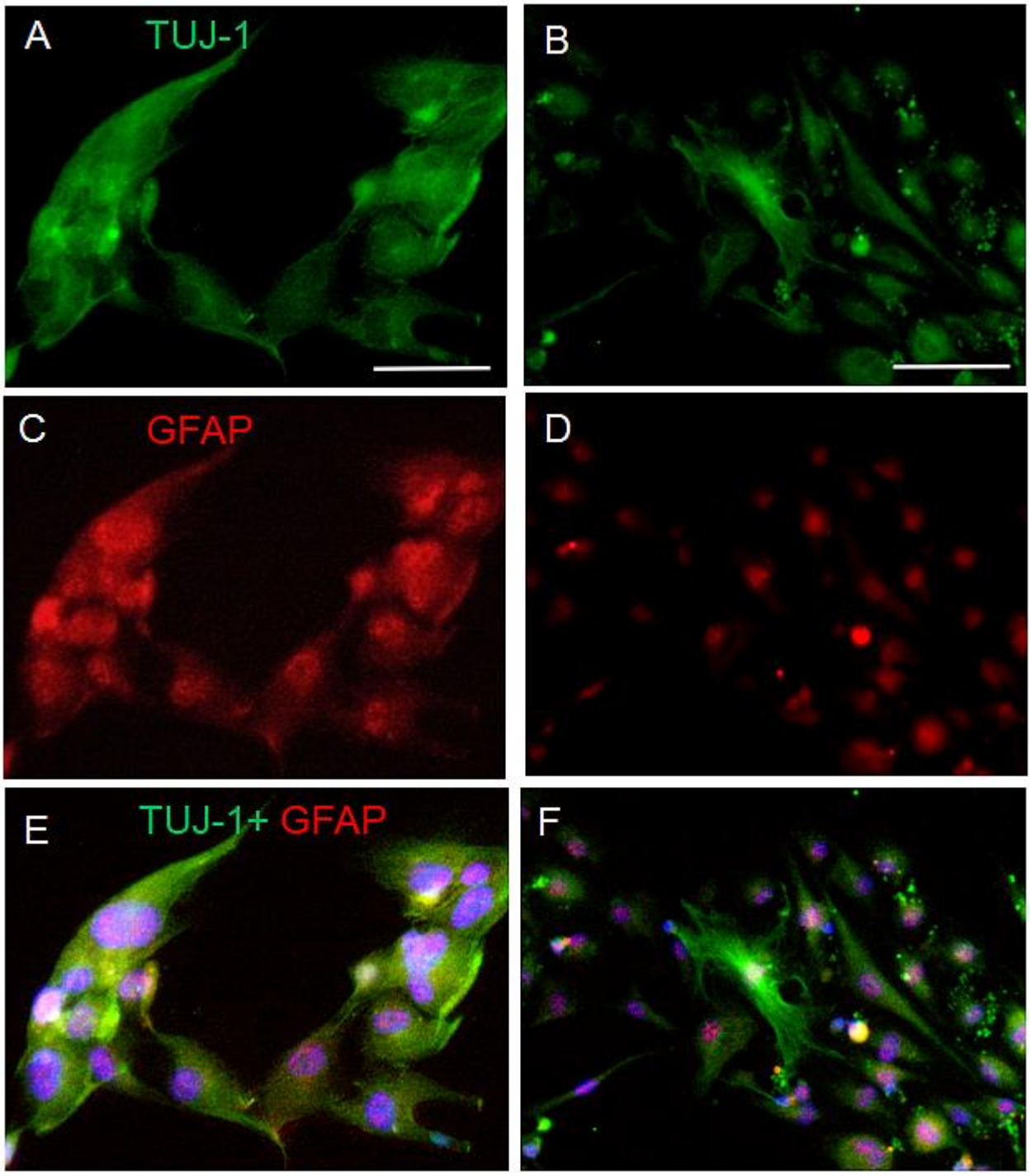

Table 1. Antibodies and Cytokines

Antibody	Species	Dilution	Company	Catalogue #	Antigen
FITC Isotype	Mouse	1:200	Biolegend	400110	--
PE Isotype	Mouse	1:330	Biolegend	400114	--
CD29 PE	Mouse	1:160	BD Pharmigen	555443	Human
CD54 PE	Mouse	1:160	BD Pharmigen	555511	Human
CD73 PE	Mouse	1:160	BD Pharmigen	555257	Human
CD90 PE	Mouse	1:160	BD Pharmigen	555596	Human
NESTIN PE	Mouse	1:160	BD Pharmigen	IC1259P	Human
CD271 FITC	Mouse	1:160	BD Pharmigen	345104	Human
GFAP	Rabbit	1:160	DAKO Cytomation	Z-0334	Human
TUJ-1	Mouse	1:160	SIGMA	T-8660	Human
P63	Rabbit	1:160	BSB-5851	BSB-5851	Human
CitK5/6	Mouse	1:160	Millipore	MAB1620	Human
HuR EGF		20 ng/ml	GIBCO	PHG0311	Human
HuR FGF		20 ng/ml	GIBCO	234 FSE	Human
Forskolin		5 µM	SIGMA	F6886	
A Trans Retinoic acid		1 µM	SIGMA	R2625	

Table 2. Primer sequences specific for neural growth factors

Primer	Sequences	Base pairs
Oct ¾	5'-GAGCAAAACCCGGAGGAGT-3' 5'-TTCTCTTTCGGGCCTGCAC-3'	310
MASH-1	5'-GCGTTCAGCACTGACTTTTG-3' 5'-CCCCGGGAGACTTCTTAGAG-3'	207
Sox-2	5'- CGGCCCCGGCGGAAAACCAA-3' 5'- TCGGCGCCGGGGAGATACAT-3'	515
BDNF	5'- AGCCTCCTCTGCTCTTTCTGCTGGA-3' 5'- CTTTTGTCTATGCCCCTGCAGCCTT-3'	298
NT4	5'- AGCGAAACTGCACCAGCGAG-3' 5'- CACCTTCCTCAGCGTTATCA-3'	202
NT3	5'- CCCGAGAGCCGGAGCGGGGA-3' 5'- GTGACTCTTATGCTCCGCGT-3'	230
β-actin	5'- TCCTGTGGCATCCACGAAACT-3' 5'- GAAGCATTTGCGGTGGACGAT-3'	340

Table 3. Expression of protein markers for Hu-OMSC

	DMEM/F12 CM			DMEM/F12-GF		
	n1/n2	**Median1 (10% FBS)**	**Percentile 25%**	**Median2 (0% FBS)**	**Percentile 25%**	***U Mann-Whitney/P***
MSC						
CD29	11/13	99	96	78	77	11.5/**0.0001**
CD73	10/11	97.5	96	61	39	0/**0.0001**
CD90	12/14	99	98	96.5	85.5	26.5/**0.0001**
HBC						
CD54	14/14	55	13	19.5	7	59.5/**0.039**
Ensheating cells						
NESTIN	11/14	89	61	93.5	60.7	65/0.26
GFAP	9/10	38	29	61.5	29	35/0.22

n: number of subjects ;

U Mann-Whitney/P: statistical value/probability