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Neural stem cell separation from the embryonic avian olfactory epithelium by sedimentation field-flow fractionation

I. Comte [a], S. Battu [b,*], M. Mathonnet [a], B. Bessette [a], F. Lalloué [a], P. Cardot [b], C. Ayer-Le Lièvre [a]

[a] *Laboratoire de Neuro-Immunologie et Développement, EA 3842 "Homéostasie Cellulaire & Pathologies", Faculté de Médecine, 2 rue du Dr. Marcland, 87025 Limoges Cedex, France*
[b] *Laboratoire de Chimie Analytique, EA 3842 "Homéostasie Cellulaire & Pathologies", Faculté de Pharmacie, 2 rue du Dr. Marcland, 87025 Limoges Cedex, France*

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Dedicated to Christiane Ayer-Le Lièvre who passed away in August 2004.

Abstract

The aim of the present study was to isolate neural stem cells from a complex tissue: the avian olfactory epithelium; by using sedimentation field flow fractionation (SdFFF). By using "Hyperlayer" elution mode, fraction collection and cell characterization methods, results shows that SdFFF could be a useful cell sorter to isolate an enriched, viable and sterile immature neural cell fraction from which the reconstitution of a complete epithelium was possible. In culture, SdFFF eluted cells first led to a "pseudoplacodal" epithelioid cell type from which derived "floating cells". These cells were then able to generate neurosphere-like structures which were composed of cell having many features of immature cells: undifferentiated, self-renewable and multipotentiality. Such a population might be used as a model to improve our understanding of the mechanisms of olfactory neoneurogenesis.

Keywords: Avian embryos; Olfactory epithelium; Neural stem cells; SdFFF; Cell separation

1. Introduction

Introduced in the late 1960s by J.C. Giddings, field flow fractionation (FFF) methodology, a chromatography-like separation method, was described as one of the most versatile particle separation techniques [1]. Like all other FFF methods, separation using SdFFF is achieved by the combined action of a parabolic flow profile, generated by flowing a mobile phase through a ribbon-like capillary channel, and of an external field applied perpendicularly to the flow direction [1]. SdFFF, also called centrifugal or multi-gravitational-FFF, uses a multi-gravitational external field generated by the rotation of the separation channel in a complex device [1–4].

SdFFF appears to be particularly well suited for purification and characterization of micron-sized particles and was adapted to cell separation [3–6]. The SdFFF elution mode for cells is described as "Hyperlayer" [1,2,7–13]. In such a mechanism, cell size, density, shape or rigidity is involved, as are channel geometry and flow rate characteristics. At constant flow rate and external field strength, larger or less dense particles are eluted first [1,2,7–13]. Thus, SdFFF device setup and elution conditions should be optimized to promote the "Hyperlayer" elution mode enhancing a better sub-population separation in association with a drastic limitation of cell–wall interactions [3]. Since the pioneering report of Caldwell et al. [2], FFF, SdFFF and related technologies have shown a great potential for cell separation and purification with major biomedical applications including hematology, cancer research or molecular biology [14–22]. In a recent paper [5], we have shown the interest in ES cell (mouse embryonic stem cells) sorting to select the most appropriate population to obtain transgenic mice. We have also opened the field of neuroscience with the purification of neurons and immature neural cell fractions without induction of cell differentiation [4,6].

* Corresponding author. Tel.: +33 5 5543 5979; fax: +33 5 5543 5858.
E-mail address: battu@pharma.unilim.fr (S. Battu).

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Actually, a wide panel of techniques and methodologies are available for cell separation and characterization: centrifugation, elutriation, electrophoresis, flow cytometry (FC) or magnetic-activated cell sorting (MACS), which take advantages of biophysical criteria (size, density, shape, ...), electrical charge or specific antigen expression [3,16,23–26]. SdFFF takes advantage of intrinsic biophysical properties of cells and combines the possibilities of flow driven separation techniques (elutriation, chromatography) and of field induced and focusing techniques (electrophoresis, centrifugation) [1,3,14,16]. SdFFF might be more useful than many other cell sorters to provide purified, viable and usable cell fractions because (1) no complex mobile phase and no long, expensive cell preparation or labeling are needed; (2) elution and sorting is very fast and gentle; (3) because the device can be easily and rapidly optimized for each new separation problem. As specific pre-labeling (fluorescent or magnetic) is not necessary (tagless method), SdFFF is an interesting method for applications in which labels could interfere with further cell uses (culture, transplantation, immuno-blotting), when labels do not exist or when labels could induce cell differentiation (for example stem cells) [3,14,16]. Nevertheless, the association of SdFFF with specific biological characterization by flow cytometry could be a very effective tool to calibrate fractograms, enhancing fraction collection and sub-population sorting [3,5,6].

In nervous system, the olfactory epithelium (OE) affords an interesting tool to study stem cells and set up isolation from different cell types. Indeed, OE is located in nasal cavity and is easily accessible to removing. The OE is a pseudo-stratified columnar epithelium consisting of four cell types: (1) sustentacular cells, (2) olfactory receptor neurons (ORNs), and two basal populations of proliferative cells, (3) the horizontal basal cells (HBCs), which lie in close apposition to the basement membrane and (4) the globose basal cells (GBCs). ORNs are continuously replaced in both immature and adult animals. Several pieces of evidence suggest that the basal cells are a self-renewing source of new sensory neurons and act as stem cells [27–29]. Olfactory stem cells might be either GBCs or HBCs subpopulations [30,31].

The aim of the present study was to isolate neural stem cells from embryonic tissues. The choice of an embryonic avian olfactory epithelium as a source of immature neural should demonstrated the capacity of SdFFF to sort, an enriched, viable and sterile population of these cells, without induction of cell differentiation. As we did to sort neuron cells [4], and in contrast to our previous work on cancer cells line [6], cell separation was performed on complex populations containing cell types which could be defined as neural stem cells.

Results showed that SdFFF allows distinguishing distinct cell types in the olfactory epithelium and also might afford a useful cell sorter to provide immature neural cell fractions which could behave similarly to stem cells leading to the reconstitution of a complete epithelium. Such a population could be used as a model to improve our understanding of the mechanisms of olfactory neoneurogenesis and could open the way for future experiments concerning the mechanisms of neuronal homeostasis.

2. Materials and methods

2.1. Establishment of primary olfactory cell culture

Fertilized chicken eggs (*Gallus gallus Linné*) from commercial sources (Commercial label chicken heterozygous for naked neck gene, Couvoir du Faget, Lot, France) were incubated at 38 °C in a humidified atmosphere. Embryos were staged according to the developmental timetable of Hamburger and Hamilton for the chicken [32].

Embryonic olfactory epithelia were dissected from chick embryos at stage ED14 when the four cells types are detectable. Tissues were incubated at 37 °C in Neurobasal™ Medium (Life Technologies, Cergy Pontoise, France) with 10% trypsin (Life Technologies). After 30 min, cells were dissociated manually by pipeting and were transferred on cell strainer (100 μm, Becton Dickinson, Franklin Lakes, NJ, USA) for a final separation. They were then plated on coverslips (Fisher Bioblock Scientific, Illkirch, France) and incubated in Neurobasal™ Medium supplemented with 10% fetal calf serum (FCS) (Life Technologies). These cultures were maintained at 37 °C in a 5% CO_2 atmosphere and the Medium was changed every 2 days for two weeks. Then the morphological differentiation of the four cell types characteristic of the olfactory epithelium could be analyzed. Staining with hematoxylin (Sigma–Aldrich, Saint Quentin Fallavier, France) was used for histological observation of these cultures.

2.2. Sedimentation field flow fractionation technique

Embryonic olfactory epithelia were dissected from at least 8 chick embryos at stage ED14. Cells were prepared as described above. The SdFFF separation device used in this study was derived from those previously described and schematized [3,4]. The separation channel was made up of two 870 mm × 30 mm × 2 mm polystyrene plates, separated by a Mylar® spacer in which the channel was carved. Channel dimensions were 785 mm × 10 mm × 0.125 mm with two V-shaped ends of 70 mm. The measured total void volumes (channel volume + connection tubing + injection and detection device) were 960 ± 5 μL ($n = 5$). Void volumes were calculated after injection and retention time determination of an unretained compound (0.1 g/L of benzoic acid, UV detection at 254 nm). The channel–rotor axis distance was measured at $r = 13.8$ cm. Sedimentation fields were expressed in units of gravity, $g = 980\ cm/s^2$, and calculated as previously described [4]. A Spectroflow 400-ABI Kratos chromatographic pump (ABI-Kratos, Ramsey, NJ, USA) was used to pump the sterile mobile phase. A M71B4 Carpanelli engine associated with a pilot unit Mininvert 370 (Richards Systems, Les Ullis, France), controlled the rotating speed of the centrifuge basket. Sample injections were done by means of a Rheodyne® 7125i chromatographic injection device (Rheodyne, Cotati, CA, USA). Cleaning and decontamination procedures, as well as devices involved in these processes, have been described in a previous report [3]. The elution signal was recorded at 254 nm by means of a Waters 484 tunable absorbance detector (Waters

Associates, Milford, MA, USA) and a 14-byte M1101 (100 mV input) acquisition device (Keithley Metrabyte, Tauton, MA, USA) operated at 2 Hz and connected to a Macintosh computer. The optimal elution conditions ("Hyperlayer" mode) have been experimentally determined and are as follows: flow injection through the accumulation wall of 100 μL epithelial olfactory cells (1.5×10^6 cells/mL); flow rate: 0.6 mL/min; mobile phase: sterile PBS pH 7.4; external multi-gravitational field strength: 20.0 ± 0.1 g; spectrophotometric detection at $\lambda = 254$ nm. Two cell fractions were collected: peak fractions 1 and 2 (PF_n), PF_1: 3 min 30 s/6 min 00 s; PF_2: 6 min 15 s/8 min 30 s. To obtain a sufficient quantity of cells for cultures and immunofluorescence studies, eight successive SdFFF cumulative fraction collections were performed. Fractions were then plated on coverslips (Fisher Bioblock Scientific) and incubated in Neurobasal™ Medium supplemented with B27 complement specific for embryonic neuron cultures (Life Technologies). This neuronal complement allowed to study the effects of different growth factors without the effects of fetal calf serum (FCS) growth factors.

2.3. Analysis of sorted cells

We detected cell apoptosis by using the Terminal Deoxynucleotidyl Transferase-Mediated UTP-Digoxigenin Nick-End-Labeling (TUNEL) technique. The labeling of the DNA break *in situ* was performed according to the "*In Situ* Cell Death Detection Kit POD" (Roche Molecular, Meylan, France), a TUNEL method described by Gavrieli et al. [33]. Labeling was revealed by diaminobenzidine (DAB, Metal enhanced Substrate Set, Roche Molecular).

Mitosis was detected by BromodeoxyUridine (BrdU) incorporation. 10 μmol/L of BrdU was added to primary cell cultures or cell sorted cultures. The culture was maintained from 4 h (to analyze the proliferation of only "floating cells") to 48 h (to analyze the proliferation of all cell types), at 37 °C in a 5% CO_2 atmosphere according to the experiments. The BrdU was detected by immunocytochemistry using a mouse anti-BrdU antibody according to the 5-bromo-2′-deoxy-uridine labeling and detection Kit II manufacturer's instructions (Roche Molecular). To detect the proliferation of the "floating cells", BrdU was added to the culture just after replating. Then, after 4 h, the incorporation of BrdU was detected and the number and the shape of positive cells were examined.

Apoptotic index and BrdU labeling index (BrI) were obtained as the ratio of the number of positive cells in five microscopic fields per treated cell culture. The measurements were repeated for 5 different cultures operated in the same conditions.

Differentiation of HBCs was analyzed by indirect immunofluorescence staining. The cells on the culture coverslip were fixed with 4% PFA (paraformaldehyde), permeabilized with Triton X100, blocked with 1% bovine serum albumin (BSA) (Sigma–Aldrich) and then incubated overnight at 4 °C with the primary antibody. Several primary antibodies were used to characterize the different olfactory cell types. The monoclonal mouse anti-TrkA (Santa-Cruz Biotechnology, CA, USA) was prepared at a 1:200 dilution. The monoclonal mouse antibody 1.1.E10, which was previously shown to recognize four chick keratins K3, K12, K14 and K18 (Dr. Dhouailly, personal communication), was used without dilution and the anti-Calmodulin dependent protein kinase type II monoclonal antibody (CamKII) (Roche Molecular) was diluted to 1:200. The Alexa fluor 488 or Alexa fluor 594 goat anti-mouse antibody (Molecular Probes, Leiden, Netherlands) was added at a dilution of 1:200 after washing and incubation in the blocking solution. Then, slides were washed and mounted with glycerol–gelatine medium (Sigma–Aldrich) and analyzed on a LSM-510 confocal microscope (Carl Zeiss, Jena, Germany).

In situ hybridization was used to detect mature olfactory neurons which expressed chick olfactory receptor 2 (COR2), COR3 and COR4. A mix of COR2, COR3 and COR4 riboprobes prepared from plasmids each containing cDNA was used as marker. Before treatment for hybridization, cells were fixed for 20 min in freshly prepared 4% PFA in PBS pH 7.5. Then they were incubated in acetate for at least 20 min.

Prehybridization, hybridization, washing and immunological detection conditions were as described previously [34]. All reagents used were molecular biology grade. Solutions were filtered and autoclaved. Finally, before observation on a microscope (Leica, Rueil-Malmaison, France) with a Nomarski contrast equipment, the cells on the culture coverslip were mounted in Shandon Immu-Mount (Thermo Electron Corporation, Pittsburgh, PA, USA).

3. Results and discussion

3.1. Olfactory cell types in primary culture

During life, olfactory neurons are continually lost owing to disease or injury. So, there is a continued turnover of neurons associated with a compensatory neurogenesis [28,29,35]. This ability to regenerate each characteristic differentiated cell type corresponds to a functional similarity between the olfactory epithelium and regenerating tissues such as liver, epidermis or blood. As with those other tissues, the presence of stem cells in the olfactory epithelium has long been hypothesized to be the basis of its regenerative capacity. Olfactory stem cells might be either GBCs or HBCs subpopulations [30,31]. GBCs can be described as an heterogeneous population that contains two cell types, some express mash 1 and the others express Ngn1 [36,37]. These two types of basal cells can be distinguished phenotypically, using cell-specific markers: HBCs are immunoreactive for keratin 5 and 14 [38–42] and tropomyosin related kinase A (TrkA) [43], whereas GBCs do not express keratin or TrkA [41,44].

In order to isolate these stem cells, the experimental model chosen for this study was the embryonic avian olfactory epithelium because of its accessibility. First, the presence of stem cells in the avian olfactory epithelium was checked by primary olfactory cell cultures. After 15 days *in vitro*, the primary olfactory cell culture was composed principally of three cell types: a fibroblastic cell type which consisted of widely spread cells, a bipolar cell type with long thin fibers and aggregates of small round cells forming neurosphere like structures (Fig. 1A). To identify these different cell types, an immunocytochemistry

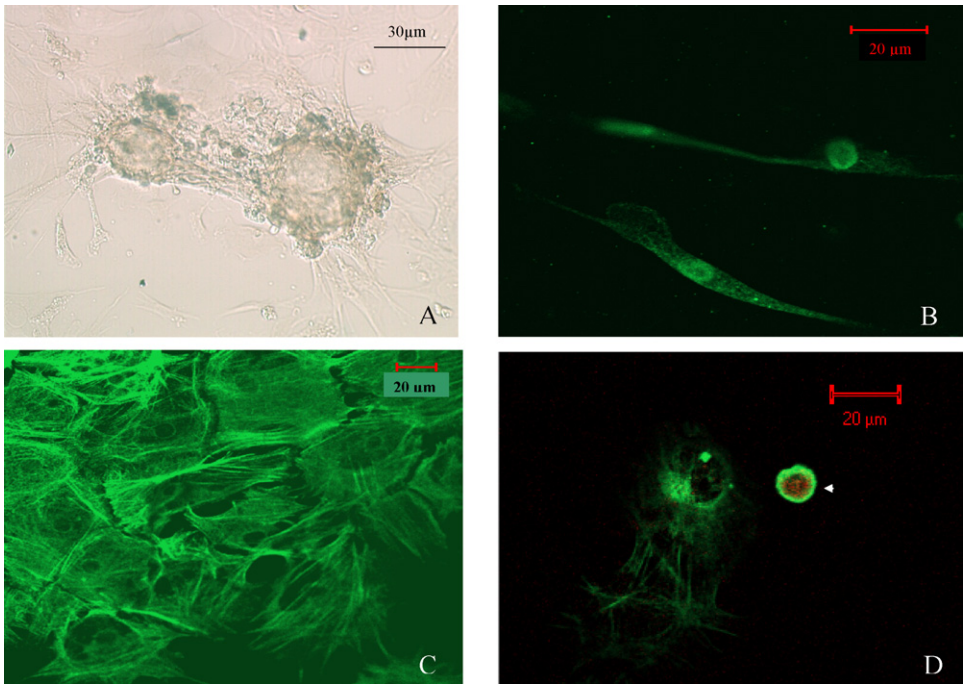

Fig. 1. Characterization of cell types in an olfactory primary cell culture after 15 days. Olfactory primary cells culture was composed of neurosphere like-structures (A), neuronal cells stained with anti-CaMKII antibody (B), sustentacular cells labeled with anti-keratin antibody (C) and HBCs expressing both keratin (green fluorescence) and TrkA (Red fluorescence) (D).

study was performed with specific antibodies. An anti-CaMKII antibody was used to detect mature neurons, an anti-keratin antibody to identify both sustentacular cells and HBCs, and an anti-TrkA antibody to more specifically characterize HBCs.

Immunocytochemistry with these different antibodies on primary cell cultures showed that the bipolar cells were labeled with an anti-CaMKII antibody (Fig. 1B), the fibroblastic cells with an anti-keratin antibody (Fig. 1C), and some small round cells in neurosphere-like structures with both anti-keratin and anti-TrkA antibodies (Fig. 1D). However, some of the small round cells which were negative for keratin were also negative for TrkA.

So, as expected in primary olfactory cell cultures, all cells constituting the *in vivo* olfactory epithelium were present: sustentacular cells corresponding to the fibroblastic cell type *in vitro* (keratin+), olfactory neurons which were bipolar cells (CaMKII+), HBCs coinciding with some small round cells in aggregates (keratin+ and TrkA+) and GBCs which were another type of small round cells localized in neurosphere like structures (keratin− and TrkA−).

3.2. SdFFF epithelial olfactory cell elution and collection

SdFFF cell separation and sorting require some specific considerations [3,4,6]. SdFFF separation should preserve (1) the cell functional integrity; (2) a high level of short and long term cell viability without induction of apoptosis; (3) the capability of maturation and differentiation of eluted cells. Finally, SdFFF cell separation must also provide high repeatability and reproducibility, maximal recovery and sterility of collected fractions, which is also essential if culture or transplantation is needed [3,4,6]. Thus, SdFFF elution conditions were selected to promote the "Hyperlayer" elution mode and reduce particle–channel wall interaction [3].

Fig. 2 displays a specific and representative epithelial olfactory cell elution fractogram obtained under optimal conditions (see Section 2): external field strength of 20 ± 0.1 g, flow rate of 0.60 mL/min. Two major peaks were observed (Fig. 2): the first corresponding to unretained species (void volume peak, $R_{obs} \approx 1/R_{obs}$; ratio of the void time versus the retention time (t_0/t_r) [12]), and the second (3 min 30 s to 8 min 30 s) corresponding to the retained species: epithelial olfactory cells with

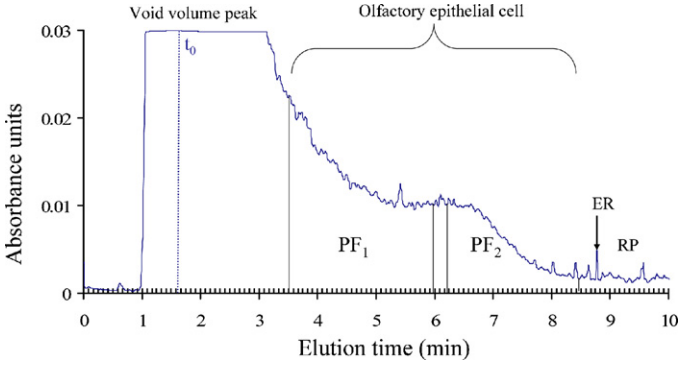

Fig. 2. Representative fractogram of epithelial olfactory cells after SdFFF elution. Elution conditions: flow injection of 100 µL of epithelial cells (1.5×10^6 cells/mL), flow rate: 0.6 mL/min (sterile PBS pH 7.4); external multi-gravitational field: 20.0 ± 0.1 g, spectrophotometric detection at $\lambda = 254$ nm. Fractions were collected as follows: PF_1, 3 min 30 s/6 min 00 s; PF_2, 6 min 15 s/8 min 30 s. ER corresponds to the end of channel rotation, in this case the mean externally applied field strength was equal to 1 g (mean gravity = 0), thus RP, a residual signal, corresponds to the released peak of reversible cell-accumulation wall sticking.

$R_{obs} = 0.297 \pm 0.004$ ($n = 15$). After total cell elution the external field was stopped (ER, Fig. 2, external field strength = 1 g, mean gravity = 0), and we observed a residual signal (RP, Fig. 2), which corresponded to cells released from the separating channel.

According to the SdFFF elution mode description [1,2,7–13], the "Hyperlayer" elution of olfactory cells was confirmed as the increase in field strength was associated with a decrease in R_{obs} values of the second peak). At a constant flow rate (0.6 ml/min), $R_{obs} = 0.297 \pm 0.004$ for a field strength of 20 g ($n = 15$), and $R_{obs} = 0.280 \pm 0.004$ for a field strength of 40 g ($n = 5$). The effectiveness of this mode to reduce particle–accumulation wall interactions is shown in part by the low level of the corresponding cell release peak at the end of the fractogram (Fig. 2). The use of these conditions in association with cleaning and decontamination procedures should allow epithelial olfactory cell elution while respecting functional integrity, viability, sterility and without induction of cell differentiation. Indeed, there were only approximately 10% of apoptotic cells after the cell separation whereas in primary culture approximately 7% of cells were apoptotic.

These conditions, in particular the very weak external field strength (20 g), were setup to specifically obtain the retention of the smaller and the denser cells of the whole olfactory epithelial population, which could correspond to neural stem cell features [45]. However, we did not know if cell separation of the different sub-populations was achieved, and in particular, if we were able to sort the population of interest from this unique elution peak (Fig. 2).

The two cell fractions respectively PF1 and PF2 were then collected and cell characterization was conducted both on SdFFF eluted fractions and on a control population.

3.3. SdFFF eluted cell culture and characterization

The PF_1 cells and PF_2 cells were plated on separate coverslips. After 10 days of *in vitro* culture, the PF_1 consisted in only cells of fibroblastic type (Fig. 3A). These cells appeared to correspond to sustentacular cells.

The PF_2 cell culture was completely different. After cell separation, PF_2 was composed of 96% small round cells labeled with the anti-TrkA antibody (Fig. 3B). After replating, all cells expressed both TrkA and keratin. At the beginning of cell culture, they looked like epithelioid cells and seemed to be relatively undifferentiated (Fig. 3C): cells were not labeled by anti-keratin, anti-CaMKII nor anti-TrkA antibodies and, morphologically, did not correspond to any cell type present in primary culture. These specific features evoked those of an embryonic placodal epithelium (Fig. 3C). When the PF2 was maintained in culture for 10 days more, some cells were detached and became "floating". Then, these "floating cells" formed aggregates and composed neurosphere like structures which settled on the coverslip. The characterization of these "floating cells" was performed by immunocytochemistry using both anti-TrkA and anti-keratin antibodies. These cells appeared to have characteristics similar to HBCs (keratin+ and TrkA+). After two weeks later in culture, they increased in number and started to differentiate, producing cell types coexisting in the olfactory epithelium *in vitro* (Fig. 1).

Thus, SdFFF appeared as an effective method to sort from olfactory epithelium an enriched fraction of immature cells from which the reconstitution of a complete epithelium was possible. These results suggest that SdFFF cell separation could permit the study of the organization and development of specific cell structures such as pseudo-placodal epithelium or olfactory epithelium.

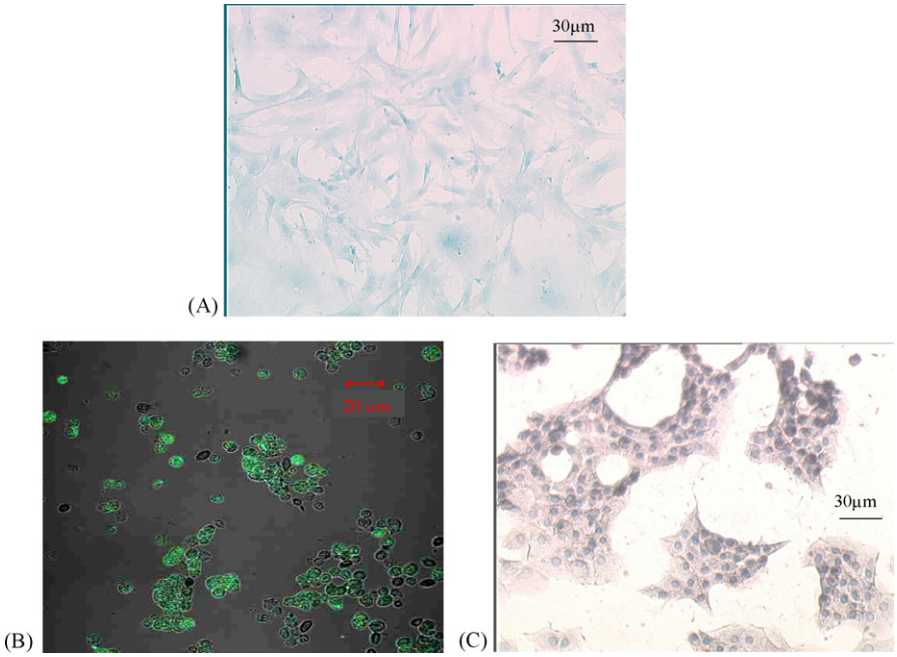

Fig. 3. Becoming of PF_1 and PF_2 cells after 10 days in culture. After 10 days in culture, PF_1 consisted of fibroblastic cells type (A); The PF_2 cells were labeled with an anti-TrkA antibody just after cell separation (B). After 10 days culture, PF_2 had an epithelioid shape. PF_2 was not labeled by anti-CaMKII, anti-keratin nor anti-TrkA antibodies (C). In order to determine their morphological aspect, PF_2 cells were stained with hematoxylin.

Cells eluted in the second fraction first gave a pseudo-placodal epithelium. Then, from this specific cellular structure, we observed the apparition of "floating cells" which were able to differentiate into the main cell types present in the olfactory epithelium. In the following part, we trigged to identify and characterize the nature and role of these "floating cells".

3.4. *In vitro characterization of "floating cells"*

Neuronal stem cells should have characteristics similar to any other tissue-specific stem cells [46]. They correspond to relatively undifferentiated cells, which regenerate their sub-population at each cell division, and also generate mature cells belonging to all lineages present in their tissue. In the case of olfactory epithelium, these mature cells are related either to neurons or to sustentacular cells and GBCs being known to belong to the neuronal lineage [36,47].

Two specific features of "floating cells" were analyzed: multipotentially and ability of self-renewal.

The multipotentiality of the "floating cells" was demonstrated by their capacity to generate all epithelial olfactory cell types. The "floating cells" were set apart, and plated on a new coverslip at different cell concentrations.

When "floating cells" were plated at a low concentration (50 cells/mL), epithelioid cells (pseudo-placodal epithelium, Fig. 3C) were observed after 10 days of *in vitro* culture.

Whereas, when "floating cells" were plated at a high concentration (5000 cells/mL), 10 days later in culture, neurosphere like structures could be observed. Two weeks later, an immunocytochemistry study was performed in which fibroblastic cell types were labeled with anti-keratin antibody (Fig. 4A), bipolar cell types with an anti-CaMKII antibody (Fig. 4B), and finally small round cells in neurospheres labeled both with anti-TrkA and anti-keratin antibodies (Fig. 4C). A small round subtype of cells which were not positive for anti-TrkA and anti-keratin labeling was also present. The presence of mature olfactory neurons was detected by *in situ* hybridization with a mix of COR riboprobes [48]. A few labeled cells with their cell body in neurospheres were observed (Fig. 4D). Thus, all olfactory cell types identified in primary cultures were also detected in these cultures: sustentacular cell types, olfactory neurons, HBCs and the presumptive GBCs. Each cell type was generated according to a specific proportion. Indeed, cultures from "floating cells" were composed of $12.3 \pm 5.9\%$ of neurons, $54.4 \pm 5.8\%$ of sustentacular cells and $24.6 \pm 5.0\%$ of HBCs. The low percentage of mature neurons is supported by the weak labeling of olfactory neurons with COR2, COR3 and COR4 after *in situ* hybridization.

With high cell concentrations, "floating cells" have the capability to proliferate highly and to give rise to different olfactory cell types.

Self-renewal was first demonstrated by the mitosis detection without growth factor. 4 h after replating and addition of BrdU, only "floating cells" could be observed and 20% of these cells had incorporated BrdU, specific of their capability for self-renewal. Considering that, in the mouse, the presumptive olfactory stem cells divide asymmetrically every 50 days, producing another stem cell and a neuronal precursor which divides rapidly several times producing many immature neurons [30],

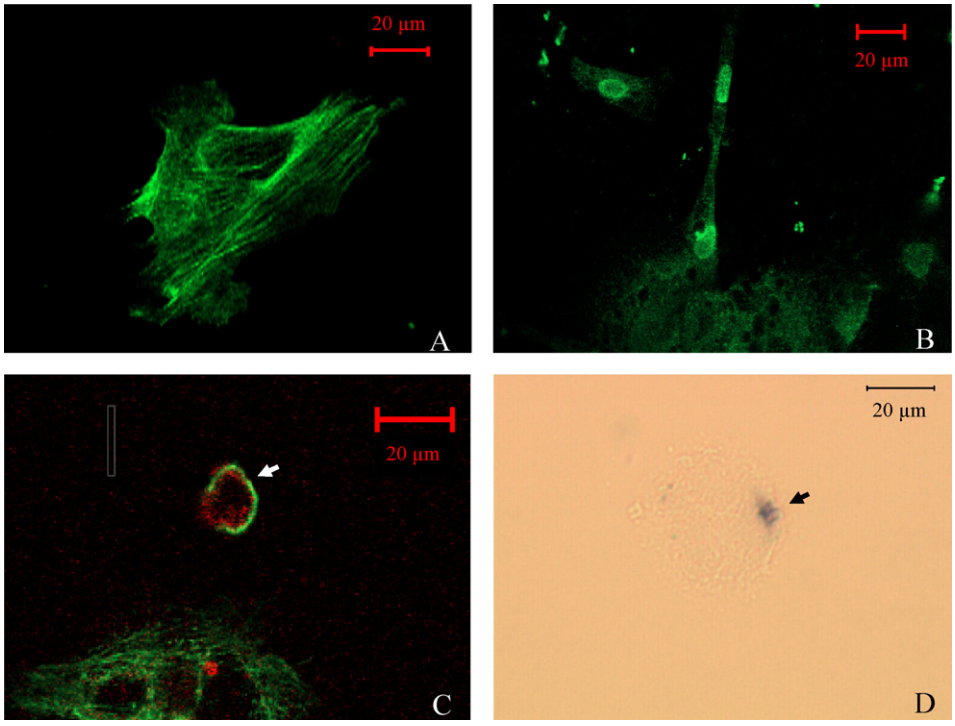

Fig. 4. Identification of cells coming from 2 weeks of high concentration "floating cells" culture. When the "floating cells", coming from the pseudoplacodal epithelioid, were replated at high concentration, different cell types were obtained. The sustentacular cells were stained by anti-keratin antibody (A). The neuronal cells were detected in these cultures by their labeling by anti-CaMKII antibody (B). Some round cells in neurospheres were labeled with both anti-keratin and anti-TrkA antibodies (C). Mature neurons were detected by *in situ* hybridization with COR2, COR3 or COR4 riboprobes (D).

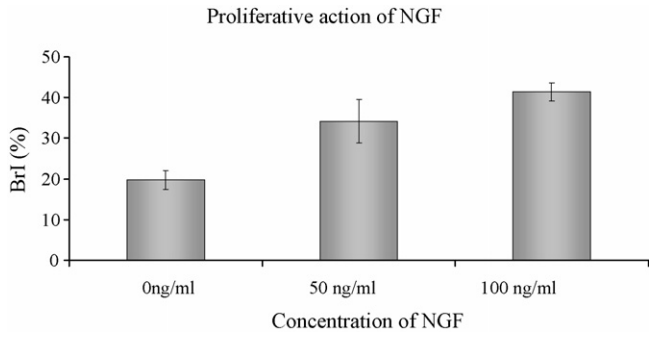

Fig. 5. Growth factors effects on "floating cells". NGF stimulates "floating cells" proliferation. Small round cells were labeled with anti-BrdU antibody after addition of 50 ng/mL and the proliferation of these cells was increased with the concentration of added NGF.

this rate of 20% of mitotically active "floating cells" is very high. Thus, "floating cells" provided by the SdFFF olfactory epithelium cell separation and having HBCs characteristics possess the critical stem cell features such as undifferentiated aspect (as defined by the lack of differentiated markers), self-renewal capacity and multipotentiality, and might be the *in vivo* dividing HBCs previously described by Carter et al. [49].

3.5. Effect of NGF on floating cells

Since TrkA, the high affinity receptor for NGF, was expressed by "floating cells", the action of this growth factor was tested on this type of culture. NGF is present in many areas of the central nervous system and both the protein and its mRNA are strongly expressed in the olfactory bulb (OB). It plays an essential role in regeneration, maintenance and development in this system of mammals [50]. Furthermore, its retrograde transport from OB to olfactory epithelium has been demonstrated [43]. The administration of NGF into the nasal cavity induces an increase of the expression of olfactory marker proteins within the olfactory epithelium of axotomized rats to compensate the effect of the olfactory neurons lesion, suggesting NGF might be considered as a trophic source for olfactory neurons [51].

So, the addition of different concentrations of NGF might increase the number of cells having incorporated BrdU in "floating cells" cultures. To determine the effect of addition of NGF on the number of generated olfactory neurons, 50 or 100 ng/mL of NGF were added in freshly started cultures of "floating cells" (Fig. 5).

The addition of NGF to the culture of "floating cells" stimulated cell proliferation (Fig. 5), but this increase in mitotic rate was not correlated with a high number of generated neurons even though there was a low increase in neuron number as demonstrated by the low percentage (30%) of cells expressing marker for mature neurons, CamKII. These results are consistent with the previous hypothesis suggesting "floating cells" are stem cells and addition of NGF would promote their proliferation to produce new stem cells and immediate progenitors [52]. However, NGF has no direct effect on immediate progenitors, which explains why their increased number is weak as well as the number of olfactory neurons to which they give rise to.

4. Conclusion

Taken together, the results of cell culture and cell characterization methods (immunochemistry, *in situ* hybridization, ...), showed that SdFFF could be used to sort specific cell populations from a complex epithelium. SdFFF eluted cells led to a "pseudoplacodal" epithelioid cell type. These cells were then able to generate neurosphere-like structures which were composed of cells presented many features of immature cells: they were relatively undifferentiated, multipotent and were capable of self-renewal.

SdFFF takes advantage of intrinsic biophysical properties of eluted cells and (1) because of no specific cell preparation or labeling are needed; and (2) because elution is very fast (less than 10 min); SdFFF might afford a useful cell sorter to provide an enriched, viable and sterile immature neural cell fractions which could behave similarly to stem cell. Such a population might be used as a model to better understand the mechanisms of peripheral neoneurogenesis and supports the hypothesis in which FFF might afford an original and interesting in order to open the way for future experiments concerning the link between neurogenesis and neuronal death in this biological system.

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