Comparison of bulbar and mucosal olfactory ensheathing cells using FACS and simultaneous antigenic bivariate cell cycle analysis

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BULBAR and MUCOSAL OECs: FACS and CELL CYCLE

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ABSTRACT

Transplantation of olfactory ensheathing cells (OECs) is a promising route for CNS repair. There have, however, been major discrepancies between the results from different groups. Part of this can be attributed to variations in cell sources and culture protocols. Accurate estimation of the proportions of OECs and their associated fibroblasts (ONFs) and their evolution with time in culture is an essential baseline for establishing the reparative properties of transplants. In this study we compare the evolution of cultures from the superficial layers of the olfactory bulb with tissue from the olfactory mucosa, both whole and split into lamina propria and epithelial layer. In all cases the relationship of antigenicity to morphology depended on plating density. We used FACS based on p75 and Thy1 to provide a robust and objective numerical estimate of the numbers of OECs and ONFs respectively in the cultures. A novel four colour simultaneous antigenic bivariate cell cycle analysis shows that proliferation of OECs is time limited, and is unable to prevent an overall loss of OECs with time. Overall the numbers of OECs in the cultures was inversely correlated with the deposition of fibronectin. Further culture of the cells purified by flow cytometry shows that, whereas the Thy1 population is terminally differentiated, the p75 population from the mucosal samples generates sub-populations with different antigenic phenotypes, including the re-appearance of a sub-population of p75 cells expressing FN. Culturing epithelial samples at high density reveals an unexpected transient stem cell like population of rapidly proliferating p75 positive cells.

INTRODUCTION

Transplantation of olfactory ensheathing cells (OECs) has been identified as a promising approach for anatomical and functional repair of injuries to nerve fibre tracts in the spinal cord, spinal roots, and potentially other CNS sites (Lindsay et al., 2010; Raisman and Li, 2007; Santos-Benito and Ramon-Cueto, 2003; Boyd et al., 2003; Franklin and Barnett, 2000; Radtke et al., 2008; Mackay-Sim and St John, 2010; Richter and Roskams, 2008). There have, however, been major differences in reported outcomes, ranging from functional repair by regeneration of severed axons to functional amelioration by enhancement of surviving tissue (plasticity, sprouting etc), as well as reports of failure to achieve benefit (Raisman and Li, 2007; Li et al., 1997; Ramón-Cueto et al., 1998; Radtke et al., 2010; Nash et al., 2002; Smith et al., 2002; Takami et al., 2002; Chuah et al., 2004; Chung et al., 2004; Radtke et al., 2004a; Guest et al., 2008; Bretzner et al., 2008; Yamamoto et al., 2009; Lu et al., 2001; Ramon-Cueto et al., 2000).

Apart from differences in lesion site and size, techniques of transplantation, and methods of functional assessment, there have been considerable differences in cell preparation (Chuah et al., 2010). Examples include: (**a**) the source of the tissue: olfactory bulb, (Gudiño-Cabrera and Nieto-Sampedro, 1996; Li et al., 1997; Ramón-Cueto et al., 1998; Franceschini and Barnett, 1996), olfactory mucosa or lamina propria; (Lu et al., 2001; Au and Roskams, 2002; Steward et al., 2006; Richter et al., 2005), (**b**) the age of the donor (adult, neonatal, or foetal; (Huang et al., 2008; Barnett and Roskams, 2008; Imaizumi et al., 1998)), (**c**) the time in tissue culture (Radtke et al., 2010; Ito et al., 2006a; Garcia-Escudero et al., 2010a; Llamusi et al., 2010; Novikova et al., 2010), (**d**) whether the OECs are purified (in most studies, e.g. (Chuah and Au, 1993; Barnett and Chang, 2004)), or used as mixed primary cultures (Deumens et al., 2006; Raisman and Li, 2007; Yui et al., 2011), or cell lines (Franklin et al., 1996; Audisio et al., 2009; Garcia-Escudero et al., 2010b), (**e**) the use of growth factors (Pollock et al., 1999; Bianco et al., 2004; Radtke et al., 2004b); (**f**) the species: apart from the rat, other OECs (Lindsay et al., 2010; Wewetzer et al., 2010) have been prepared from mouse (Richter et al., 2008), pig (Radtke et al., 2004b), dog (Ito et al., 2006b; Krudewig et al., 2006; Ito et al., 2006a), monkey (Guest et al., 2008) and human (Féron et al., 1998; Barnett et al., 2000; Lim et al., 2010; Gorrie et al., 2010).

Accurate estimation of the proportions of OECs and olfactory nerve fibroblasts (ONFs) and their evolution with time in culture is essential to provide a baseline for establishing the reparative properties of transplants. Primary cultures, however, are heterogeneous, with overlapping cells and wide regional variations, so that cell counting based on immunolabelling is laborious and inevitably has a subjective element. To obtain more objective estimates we therefore carried out flow cytometric analysis of the evolution of the relative populations of OECs and ONFs cultured from tissue samples taken from the adult rat olfactory bulb (OB), the whole olfactory mucosa (WM), the mucosal lamina propria (LP) and a hitherto unexplored source of OECs, the olfactory mucosal epithelium (EPI). A novel four colour simultaneous antigenic bivariate cell cycle assay was used to determine the cell cycle kinetic distribution of the antigenically identified cells in the primary cultures and to explore the relationship of proliferation to the evolution of the population changes in the cultures. The further behaviour of the sorted cells was examined in post-purification culture.

RESULTS

Histology

Olfactory ensheathing cells (OECs) accompany the olfactory nerve axons during their entire course from the olfactory mucosa to the olfactory bulbs (Doucette, 1984; Raisman, 1985; Choi et al., 2008).

In the olfactory mucosa OECs are located at the points where the olfactory axons penetrate the basal lamina interface between the olfactory epithelium and the lamina propria (Choi et al., 2008), where they ensheathe the massed bundles of olfactory nerve fibres. They are asymmetrical cells which on one surface face the olfactory nerve fibres, and on the other, face encircling fibroblastic processes from which they are separated by a basal lamina. OECs express the common glial antigen S100β (Fig. 1A) throughout the entire cell including the fine processes which are interposed among the olfactory nerve

fibres (Turner and Perez-Polo, 1992). In contrast, the p75 antigen is expressed only on the circumferential OEC outer membranes facing the encircling fibroblast-apposed basal lamina, but is virtually absent from the inner OEC membranes interposed among the nerve fibres (Fig. 1B).

In the olfactory bulb the OECs in the olfactory nerve layer express S100β but with this antibody they are indistinguishable from the S100β+ astrocytes of the glomerular and deeper layers (Fig. 1C). p75 is hardly detectable in any of these layers of the intact olfactory bulb (Fig. 1D).

Culture

Olfactory ensheathing cells (OECs) and olfactory nerve fibroblasts (ONFs) were studied in cultures of tissue samples from the outer nerve fibre and glomerular layers of the olfactory bulb (OB), and from the olfactory mucosa taken either as a whole thickness sample (WM), or separated into its component lamina propria (LP) and epithelium (EPI). The characteristics of the cell populations cultured from these 4 sources will be described in terms of:

I morphology, II immunocytology, III quantification by flow cytometry, IV analysis of cell proliferation, and V further culture of FACS purified cells.

A note on terminology: In cultures from the olfactory system the histological entities represented by olfactory ensheathing cells (OECs) and olfactory nerve fibroblasts (ONFs) are generally identified as p75+, S100β+, Thy1-, FN- and p75-, S100β-, Thy1+, FN+, respectively. For convenience in reading, in this account, we will use the terms OECs and ONFs simply to indicate these phenotypes, but with the *provisos* that the expression of p75 is regulated, and not all p75+ cells are necessarily OECs. Some cells are positive for both markers and, as will be seen below, and cells purified on the basis of p75+ can give rise to Thy1+ cells on subsequent culture. We have chosen the term ONF's, rather than simply 'fibroblasts' in order to distinguish them from fibroblasts in other areas. It must also be borne in mind that the ONFs associated with the nerve bundles in the LP may differ from those associated with the nerve fibre layer in the OB.

I Morphology

At initial plating the cultures consisted of free-floating rounded cells with no processes. From 3 DIV in the WM and LP cultures cells became adherent. In the case of OB and EPI cultures significant amounts of cellular material and debris were lost at each media change and the cells only became adherent at around 5 DIV. From 8 DIV, the morphology of the cells in all 4 cultures varied depending on the initial plating density. Plating density was taken as optimal when the cells separated into two distinct populations of spindle shaped cells and flattened multipolar cells, which corresponded antigenically to cells with OEC and ONF markers respectively (see below) but with no signs of senescence. Trial of different plating densities (see Material and Methods) showed that this result was achieved for the OB tissues by plating the samples from two olfactory bulbs on a 60 cm^2 plate, or for WM and LP by plating at an initial density of 27,000 cells/cm^2, but EPI tissues required a ten-fold increase in plating density to 270,000 cells/cm^2. For tissues from all 4 sources plated at lower densities the cultures failed to become confluent, and there was a change in cell morphology. At around 8 DIV, there was a spectrum of cell shapes ranging from spindle shaped cells with expanded ends to flattened multipolar cells. By 14 DIV the

majority of cells had become multipolar. Conversely, in WM and LP cultures plated at higher density (81,000 cells/cm^2) the majority of the cells adopted a spindle shaped morphology.

II Immunocytology

In culture we identify the OEC phenotype as positive for p75, S100β and nestin and negative for Thy1 and fibronectin (FN). ONFs are positive for Thy1 and FN, and negative for p75, S100β, and nestin. With our current matching of primary and secondary antibodies cultures can be double immunostained either using p75 together with FN, or S100β together with Thy1.

In cultures from all 4 sources at their respective optimal densities, immunocytochemistry showed (a) cells which were positive for the OEC markers p75 (Fig. 1 E1-4,9-12.) and S100β (Fig. 2 A1,3, C1,3), (b) cells positive for the ONF markers FN (Fig. 1 E5-8, 9-12) and Thy1 (Fig. 2 A2,3, C2,3), and (c) a small population of p75+FN+ cells in the WM cultures appearing from 7 days in culture (Fig. 1 E10). Fig. 2 B1-3, D1-3 shows the comparison between FN staining, which is initially associated with the ONF cell bodies and later becomes increasingly deposited in the extracellular matrix, and immunostaining of Thy1, which is a membrane associated glycoprotein (punctuate at high power) anchored to the cell surface of the ONFs.

With increasing time in culture the numbers of OECs fall dramatically (Fig. 2 E1,3, F1,3) and the FN staining, initially around cell bodies becomes widely spread over the matrix (Fig. 2 E2,3, F2,3). By 4 weeks, especially in the cultures started at lower densities, a number of the p75 and Thy1 cells are senescent, with greatly enlarged, flattened shapes with abnormal cytoplasmic granular and vacuolar inclusions, and long thin filamentous processes (Fig 2 G,H).

III Quantification by flow cytometry

Fig. 1E illustrates the difficulty in counting cell subpopulations in mixed cultures. To provide a more reliable assessment of cell numbers the cell surface markers p75 and Thy1 were used for flow cytometric analysis of cells dissociated from all 4 tissue sources plated at their relevant optimal densities (see above) either without culture (0 DIV) or cultured for 7, 10 and 14 DIV. After gating, the cytograms confirm the immunocytochemical observation of the development of distinct, non-overlapping populations of p75+Thy1- (OECs) and p75-Thy1+ (ONFs), as well as an uncharacterized double negative (p75-Thy1-) population. In addition the WM cultures also developed a small population of double positive ($p75^{+}Thy1^{+}$) cells which were not seen in the cytograms of cultures of the bulbar tissue or the separated mucosal EPI and LP samples (Fig. 3).

The salient features arising from this quantitative analysis are that in the OB cultures OECs predominate over ONFs, and the reverse in the WM cultures. However, cells in the split mucosal cultures behave quite differently. Unexpectedly, in view of its high proportion of OECs in *in situ* histology (Fig. 1A,B) LP samples generate the lowest proportion of OECs (less than 20%) of any sample. In striking contrast, EPI samples (with no detectable OECs histologically; Fig. 1A,B) generate the highest proportion of OECs (over 60%) of any tissue sample at 7 DIV, falling rapidly at 10 to 14DIV (Table 1, Fig. 4).

IV Analysis of cell proliferation

To investigate the extent to which proliferation contributes to the observed population changes, we used a novel four colour simultaneous antigenic bivariate cell cycle assay with EdU as the S-phase marker to characterize the dynamics of cell proliferation separately within the individual OEC and ONF subpopulations of the OB, WM, LP and EPI cultures at the different times in culture (Fig. 5 shows an typical example for a single population of OECs).

The numerical data (Table 2, summarised in Fig. 6) show that the majority of cells in all four cultures were quiescent (G0/1-phase of the cell cycle) over the 7-14 DIV period. The G2/M-phase (the mitogenic state) consistently had the lowest percentage. For the S-phase, the most striking features were in the EPI cultures (reflected to a lower extent in the WM cultures) which exhibited a high percentage of OECs in S-phase (DNA synthesis) at 7 and 10DIV, falling sharply by 14DIV, and a high percentage of ONFs in S-phase at 7DIV. In the WM cultures there was a high proportion of the doubly labelled (p75+FN+) cells in S-phase at 10DIV, rising further at 14DIV. Apart from these, the remaining OB, WM and EPI cultures showed low proliferative activity (S-phase percentage).

Correlation of Relative Population Size with Cell Proliferation

Pooling all the individual data points from each of the 4 tissue sources and each of the 3 time points the percentages of the mixed cell population that were OECs (p75+Thy1-) and the percentages that were ONFs (p75-Thy1+) were plotted against the percentages of OECs and ONFs that were EdU labelled (i.e. in S-phase). Plotted in this way, neither the proportion of cells that were OECs nor the proportion of cells that were ONFs were correlated with the numbers of OECs or ONFs that were in S-phase (Pearson's correlation coefficient, $r=0.511$, $p\leq0.001$ for the OECs and $r=0.095$; for the ONFs) (Fig 7).

However, taking the OB and the mucosal cultures (pooled WM, EPI and LP) separately (Fig. 8) revealed a highly significant positive correlation for the pooled mucosal cultures at all time points between the numbers of OECs and the proportion of the OECs that were in S-phase ($r=0.903$, $p<0.001$). For the OB cultures there was no significant correlation between the numbers of OECs and the proportion of OECs in S-phase at any of the time points ($r=0.167$). There were no significant correlations for any of the ONF populations with the proportion of ONFs in S-phase for either the bulbar or the mucosal EPI or LP cultures.

V Further culture of FACS purified cells

To examine the phenotypic stability of the FACS purified populations, cells sorted from the OB and WM tissue samples were maintained for an additional 4 DIV and then examined by both morphology and immunocytochemistry (Fig. 2I-L). At this time the majority of the FACS purified $p75^{+}Thy1^{-}$ cells retained their p75 immunoreactivity and adopted the spindle shaped morphology comparable to the OECs in the heterogeneous (unsorted) cultures (Fig. 2I,J).

Unexpectedly, however, the cultures of p75+ cells sorted from the WM sample now consistently generated (a) small clusters of p75-FN+ immunoreactive cells scattered among the majority p75+FN- population (not shown), and (b) cells which were doubly labelled for p75 and FN (Fig. 2J) , white arrow heads).

Unlike the purified p75+FN- population, however, the p75-FN+ sorted cells from OB and WM showed no antigenic changes (Fig. 2K,L). They expressed FN and adopted the multipolar morphology observed in the ONFs of the unsorted heterogeneous cultures.

DISCUSSION

Histologically, OECs *in situ* are clearly defined by their anatomical relationships (Fig. 1 A-D). During development or after lesions, the expression of p75 on the OECs in the olfactory mucosa and bulb is down-regulated, as in the case of Schwann cells (Jessen and Mirsky, 1997), by contact with axons (e.g. (Wewetzer and Brandes, 2007; Gong et al., 1994; Turner and Perez-Polo, 1994)). In the olfactory nerve fibre layer of the intact adult OB only weak p75 immunoreactivity is seen on the surface of the OECs (possibly related to axonal turnover). In the mucosa p75 appears on the outer surfaces of the OECs, facing fibroblasts, but not on the inner surfaces facing axons. This pattern suggests that p75 may be trafficked selectively to the OEC surfaces facing basal lamina/fibroblasts, but not to the surfaces facing the olfactory nerve fibres.

Transferring the OECs to tissue culture places them in an entirely different situation. Neuronal cell bodies and nerve fibres do not survive, and the OECs lose their asymmetry and their encircling form. The present FACS analysis of the primary dissociated cell cultures shows that in the mucosal tissue samples over 90% of the cells are initially negative for both p75 and Thy1. In contrast, in the bulbar tissue samples over 90% of the cells are initially p75-Thy1+ in culture, but have upregulated p75 at 7DIV. It is unlikely that trypsinization of the bulbar tissue is responsible for the absence of p75 expression since (1) p75 is also lost in mucosal preparations that are not trypsinized, and (2) after the later onset of p75 expression in culture, trypsinization at 7, 10, and 14 DIV causes no loss of p75.

In this paper we have followed the generally accepted assumption that the p75+ cells in culture are the equivalent of the anatomically defined OECs *in situ*. However, there are cautions. The regulated pattern of expression of p75 *in situ,* its early disappearance and later re-appearance in culture, and the appearance

of FN expression in cultures of sorted purified p75+ cells raise questions about what exactly is the relationship between the OECs *in situ* and the cells on which p75 appears after 7 days in culture.

Bulbar and Mucosal OECs

For clinical purposes obtaining adult OECs for autografting by an intranasal approach to the olfactory mucosa would be preferable to the more invasive procedure of obtaining bulbar OECs by craniotomy. At the genomic level, the transcriptome profiles of purified OEC from olfactory bulb and the mucosal lamina propria revealed a pattern of differentially expressed genes, with the suggestion that bulbar OECs generally express genes associated with nervous system development while mucosal OECs express genes associated with wound healing and extracellular matrix regulation (Franssen et al., 2008; Guerout et al., 2010).

On morphological and p75/Thy1 antigenic grounds we find no distinction between mucosal and bulbar OECs in our cultures. However, it is striking that cultures of tissue samples from the olfactory bulb consistently give a superior yield of p75+ cells than those from the mucosa. Our FACS analysis shows considerable differences between the OB, WM, EPI and LP cultures over 7-14 days (Figs. 2,3). Most notably, a double positive (p75+Thy1+) population was present in the WM cultures over 10 to 14 days but was absent from the OB cultures. In the cell cycle analysis the bulbar cells exhibit the general somatic cell cycle pattern (Harper et al., 2010). However, in the mucosal cultures (WM and EPI, but not LP) the level of EdU incorporation shows a proportion of p75+Thy1- cells in S-phase is as high as in embryonic and neural stem cells (Booth et al., 2008; Turner and Perez-Polo, 1992; Werbowetski-Ogilvie et al., 2009).

Cultures from both sources include ONFs. It is a common observation (e.g. (Au and Roskams, 2003; Jani and Raisman, 2004; Nash et al., 2001; Novikova et al., 2010)) that the evolution of cultures involves a time dependent decrease in OECs and a corresponding increase in ONFs. At longer times the ONFs

completely predominate in the cultures, and p75+ cells disappear. In the present study there was a strong inverse correlation between the proliferation of p75+ cells and the deposition of FN (an index of cumulative ONF activity).

When the mucosal cells FACS purified to 98% for p75 were cultured for a further 4 days, 74-85% of the population of p75+ cells were immunostained for FN (Fig. 2J), a level of expression which is beyond what could be the result of contamination. This population is comparable to the doubly labelled p75+Thy1+ population seen at 10 and 14 days in the FACS analysis of the WM samples, and was absent from the bulbar samples (Fig. 2I). In contrast, in both bulbar and mucosal samples the population of cells FACS purified for Thy1 remains antigenically unchanged after 4 post-purification days in culture, implying that the p75-Thy1+ (ONF) phenotype is a terminally differentiated character under these culture conditions.

There are a number of studies showing functional differences between the behaviour of mucosal and bulbar cells in culture (see Introduction). Using OEC-axon confrontation assays, Windus et al described the difference in lamellipodial behaviour between LP OEC and OB OEC and demonstrated that contact with axons led to adhesion of LP OECs, while OB OECs exhibited a mixture of adhesion, repulsion and parallel migration (Windus et al., 2010).

Transplantation of both mucosal and bulbar OECs have beneficial effects on damaged CNS tissue (see Introduction). However, only bulbar OECs have been reported to provide a pathway for the originally cut axons to regenerate across the lesion and restore connections with their original targets (e.g. (Li et al., 1998). The differences between the behaviour of bulbar and mucosal OECs after transplantation may reflect a cell autonomous difference between OECs from these two sources. However, there are a number of other possible factors:

(a) The percentage of OECs in the cultures. In experiments comparing transplantation of bulbar and mucosal cultures into corticospinal tract lesions we found that only bulbar cells induced axon regeneration across the lesion (Yamamoto et al., 2009). However, the bulbar cultures were far richer in OECs than the mucosal cultures (45.86 % *vs* 16.99%),

(b) The nature of accompanying cells. Astroglia and central neurons are present in the primary bulbar tissue samples. In contrast, the mucosal tissue samples include epithelial and glandular cells, and mesenchymal stem cells (Tome et al., 2009).

An uncharacterised source of OECs

A surprising finding of the present study was the generation of a rapidly proliferating population of p75+ spindle shaped cells in cultures of samples from the isolated EPI. In conditions of high density (270,000 cells/cm^2 for the EPI compared to 27,000 for the WM) we observed a striking appearance of 64.80% of p75+ cells with a high proliferation rate (56.30%) in the EPI samples at 7 DIV. The source of these cells is not clear. It is unlikely to be due to contamination by a small number of LP cells adhering to the EPI as it is separated, because cultures of entire LP samples provide a notably low yield (1.14%) of p75+ cells with a low proliferation rate (7.93%). The WM cultures, as would be predicted, had an intermediate proportion (33.58%) of p75+Thy1- cells. Both the high proportion of p75+Thy1- cells in the EPI cultures and also the low proportion (1.14%) in the LP cultures were unexpected because histologically the LP is rich in OECs *in situ* and, conversely, there are no clearly identified OECs in the epithelial layer (Fig. 1A,B).

Our results point to the presence in the EPI of an adult progenitor/stem cell capable of giving rise to p75+ cells and proliferating in culture at a rate comparable to stem cells. This rapidly dividing population is transient, and has virtually disappeared by 14 DIV (Fig. 6). The failure to maintain division probably

reflects that our culture conditions favour differentiation (Murrell et al., 2005). Multipotent stem cells, have been identified in whole olfactory mucosa (Murrell et al., 2005) and assigned to a putative lineage of horizontal to globose basal cells in the epithelial layer (Carter et al., 2004; Holbrook et al., 1995). One or both of these cells probably corresponds to the cell giving rise to the p75+ population of the present study. Although further data would be needed to demonstrate a direct lineage relationship, our evidence for a transient stem like, rapidly proliferating source of p75+ cells in stripped epithelial cultures points to the possibility of a regulated scaling up of the OEC population to produce the larger amounts of cells which would be needed in repairs of clinical spinal cord lesions.

MATERIAL & METHODS

Primary olfactory cell preparation and cell culture

All animal procedures were performed in accordance with the UK Home Office regulations and guidelines. Female adult Albino Swiss rats (200-250g) were terminally anesthetized and the heads were removed from the body for tissue collection in sterile condition.

Adult Olfactory Mucosa**:** Olfactory mucosa was collected from the upper posterior surface of the nasal septum. The area with yellow colouration closest to the cribriform plate was separated from anteriorly placed creamy coloured respiratory epithelium using a scalpel blade and collected into the complete culture medium made up of 10% foetal bovine serum (FBS) in DMEM/F12 supplemented with 1% insulin-transferrin-selenium and 1% penicillin-streptomycin (all from Invitrogen, UK). The mucosal tissue was then washed twice in ice-cold Hank's balanced salt solution ([-] $CaCl_2$, [-] $MgCl_2$) supplemented with 1% penicillin-streptomycin (HBSS; Invitrogen) to remove excessive mucus.

1) Whole mucosa (WM): the washed WM tissue was spread on a sterilized PVC cutting table disk and chopped at the thickness of 0.1mm using a McIlwain tissue chopper. The WM fragments were collected into a solution of 0.05% collagenase (Type I, 2.5g/ml; Sigma-Aldrich UK) in the complete medium and after incubating at 37°C for 5 min triturated consecutively10-15 times using a 1000µl pipette and 5-8 times a 200µl pipette into cell suspension.

2) Lamina propria (LP) and epithelium (EPI): Based on (Féron et al., 1999) and (Li et al., 2008), WM was incubated in dispase II solution (2.4 unit/ml; Roche UK) for 45 min at 37°C and the superficial EPI layer and the underlying LP layer were gently separated using a microspatula under a stereo microscope. LP tissue was transferred into HBSS to rinse off the remaining dispase, spread out on the cutting disk and chopped at the thickness of 0.1mm. The chopped LP fragments were further digested with collagenase and triturated into a cell suspension as with WM culture. The remaining EPI tissue was triturated into cell suspension in the dispase solution before they were transferred and washed in the whole medium.

WM and LP cells were seeded at the density of 27,000 cells per cm^2 in 60mm poly-L-lysine (PLL) coated dishes and EPI cells at a much higher cell density of 270,000 cells per cm^2, as lower cell density for EPI culture has been shown to cause senescence (Rubio et al., 2008).

Adult Olfactory Bulb: Culture was established as described in (Li et al., 2003). Through a dorsal craniotomy, the olfactory bulbs were severed from the brain and lifted off the cribriform plate into ice cold HBSS. Under a sterile environment on a cold plate, the meninges around the bulb were carefully peeled off, the olfactory nerve fibre and glomerular layers were dissected out and cut into $2mm^2$ fragments which were then incubated in 0.25% trypsin/EDTA solution (TE; Invitrogen) and DNase I (Sigma) at 37°C for 10 min with intermittent trituration to facilitate the enzymatic activity. After triturating the tissue by passing them 5-8 times through a series of fire-polished Pasteur pipette tips,

stopping trypsinization by adding the whole medium, spinning down and discarding the supernatant the precipitated cell pellet was re-suspended in the whole medium. The cells from 2 bulbs were seeded on to a 60mm poly-D-lysine (PDL) coated dish.

All cultures were maintained in the whole culture medium in a humidified incubator enriched with 5% CO_2 at 37°C and the culture medium was replaced every 3 days. At the first two medium changes the cell containing supernatant was spun down, and the cells re-suspended in fresh medium and replated.

Immunocytochemistry

For immunocytochemistry cells were fixed with 4% paraformaldehyde for 20min, washed three times in phosphate-buffered saline (PBS; TAAB, UK), permeabilised and blocked with 2% skim milk in PBS containing 0.1% Triton X-100 (TAAB). Primary antibodies were applied for two hours at room temperature (RT) or overnight at 4°C. Cells were washed three times and incubated with appropriate species fluorescent secondary antibodies for an hour at RT in the dark. After washing twice, cells were counter-stained and mounted using ProLong® Gold Antifade with DAPI (Invitrogen).

Primary antibodies were 1:250 mouse anti low affinity nerve growth factor receptor (anti-p75; clone 192-IgG Chemicon, Millipore, UK), 1:200 mouse anti-Thy1 (IgG clone MRC OX-7; AbCam, UK), 1:500 rabbit anti-S100β Ig (Dako, UK), 1:1000 rabbit anti-fibronectin Ig (FN; Dako), and 1:200 mouse anti-Nestin (clone rat-401; Chemicon, Millipore). Secondary antibodies were Alexa Fluor 488 and Alex Fluor 546 chicken anti-mouse IgG, Alexa Fluor 546 goat anti-rabbit IgG, and Alexa Fluor 488 donkey anti-goat IgG (all 1:400; Molecular Probes, Invitrogen).

Flow Cytometry: cell analysis and fluorescent activated cells sorting (FACS)

Flow cytometry was based on two surface markers, p75 and Thy1 (Nash et al., 2001; Au and Roskams, 2003; Ramer et al., 2004; Rege and Hagood, 2006; Soleimani et al., 2008).

Cultures were washed three times with PBS and incubated in TE for 5min to lift the cells from culture dishes. TE activity was inactivated by adding the complete culture medium. Cell clusters were triturated to obtain single-cell suspension and washed with ice-cold sterile PBS. A small aliquot of cells was counted using Trypan Blue and the concentration adjusted to 10^6 viable cells per ml for flow cytometric analysis and 10^6 cells per ml for flow cell sorting.

For flow cytometric analysis, cells were fixed with freshly prepared cold 2% paraformaldehyde and washed in PBS prior to blocking with fresh cold 5% bovine serum albumin (BSA) and 2% FCS in PBS. The cultures were incubated with mouse anti-p75 (1:150, Chemicon) diluted in 3% BSA and 2% FBS in PBS for 45 minutes in the dark on ice, washed and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:200, Molecular Probes, Invitrogen) in the dark on ice 30 minutes. The cells were washed twice to remove excessive antibody. Single labelled cells were used to calibrate the gating.

For dual labeling cells singly labelled for p75 as above were further blocked using mouse serum overnight at 4°C followed by mouse anti-Thy1 IgG, clone MRC OX-7, conjugated with phycoerythrin (Thy1-PE, 1: 150; AbCam, UK) for 45 minutes on ice in the dark. After a quick wash with ice-cold PBS, the cells were re-suspended in cold 3% BSA, 2% FCS, and then filtered using 40-μm cell strainers (BD Biosciences), ready for analysis or sorting (without paraformaldehyde fixation steps).

Cell analysis of the fixed labelled cells was performed on a BD LSR II Flow Cytometer with FACSDiva software. Cell sorting of the living cells was performed on a MoFlo XDP (Beckman Coulter) with Summit software. 70-μm nozzles were used at sheath pressure of 30 psi and flow rate of 250-400 events

per second with the sort precision mode set for purity, and plate voltage at 5.0. A total of 30,000 events were acquired. Live cells were gated using forward- and side-scatter parameters. The respective gating for the fluorescent signals is shown in Fig. 5.

Cells sorted on the basis of p75 or Thy1 were collected within defined gates based on appropriate positive and negative controls (Fig.5B and legend). Cells were collected in the complete culture medium and a small aliquot put through a verification sorting, confirming 95-98% purity. Cells meeting this criterion were centrifuged, re-suspended in medium and plated on to chamber slides (NUNC Labtek II-CC^2) coated with PLL (for mucosal cells) or PDL (for bulbar cells). After a further culture for 4 days the FACS sorted cells were fixed, and labelled using antibodies for p75 and FN.

Flow cytometric bivariate simultaneous antigenic and cell proliferation assay

Click-IT™ EdU flow cytometry assay (Pacific blue kit, Invitrogen) was used for simultaneous identification of cell cycle markers in antigenically labelled OECs and ONFs after 7, 10 and 14 days in the primary cultures (approx 10^6 cells).

1. The cultures were incubated for one hour with 5-ethynyl-2´-deoxyuridine (EdU) at a final concentration of 10μM, harvested with 0.25% TE, then inactivated with the complete culture media.

2. p75 antigenic surface marker labelling of OECs was performed as described above.

3. The cultures were fixed with Click-IT fixative and permeabilized using Click-IT saponin-based permeabilization solution. EdU was labelled with Pacific Blue azide. After removing RNA by incubation with RNAse A, total DNA was detected by staining with CellCycle 633-red.

4. Thy1-PE antigenic surface marker labelling of ONFs was performed as described above.

The cultures with these four simultaneous fluorescent labels were analyzed on a BD LSRII cytometer with parameters as above. The bivariate cell cycle profiles of the $p75^{+}Thy^{-}$ (OECs) and p75-Thy1+ (Flbs) populations were visualized based on the Pacific Blue-tagged EdU signals and the CellCycle 633 red-tagged DNA signals. After gating (Fig. 5D and legend), G0/1-phase, S-phase and G2/M-phase were plotted to determine the cell cycle kinetics of each specific subpopulation of cells.

Confocal Microscopy and Digital Image Analysis

Confocal images of fluorescent labeled cells were captured using LSM 510 Meta, a dual scanner and two-photon microscopy (Zeiss, Jena, Germany). Laser power and wavelength intensity settings were kept consistent across all the randomized micrographs taken in each session. Images were exported using LSM Image Browser software. Methods for digital image analysis using NIH ImageJ were described in (Pool et al., 2008) with slight modification. Briefly, all images were pre-processed based on the following sequential steps: color-channels splitting, thresholding, watershedding and despeckling. For nuclei counting (DAPI stack), “analyze particles” plugin was used to count the total number of cells in that image. In the p75 stack, OEC numbers were estimated using “ITCN” plugin (developed by Center for Bio-image Informatics, UC Santa Barbara, USA). Fibronectin (in the FN stack) was estimated from measuring the total surface area, multiplied by the intensity (obtained from plot contour plugin).

Histology

Tissues were fixed by perfusion with 4% paraformaldehyde, 10μm cryostat cross sections were taken through the superficial layers of the olfactory bulb and the olfactory mucosa and immunostained with S100β and p75 as above.

Statistical Analysis

Statistical analyses of data were performed with SPSS Statistic 17.0 (SPSS Inc., IBM, UK). For correlation analysis, Pearson correlation coefficient was used for n>30, Spearman rho coefficient for n<30, significance by two-tailed Student t test.

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