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In vivo Growth and Differentiation of Canine Olfactory Bulb-Derived Neural Progenitor Cells under Variable Culture Conditions

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Abstract

The dog serves as a large animal model for multiple neurologic diseases that may potentially benefit from neural progenitor cell (NPC) transplantation. In the adult brain, multipotent NPCs reside in the subventricular zone and its rostral and caudal extensions into the olfactory bulb and hippocampus. The olfactory bulb represents a surgically accessible site for obtaining cells for autologous NPC transplantation. To model conditions that would occur for ex vivo gene therapy in the postnatal brain, NPCs were isolated from the canine olfactory bulb, expanded ex vivo under different culture conditions, and compared quantitatively for growth and immunophenotype. Under standard growth conditions, canine olfactory bulb-derived NPCs (OB-cNPCs) could be expanded nearly 500-fold in the time evaluated. Canine OB-cNPCs grown on poly-D-lysine (PDL) or on PDL-fibronectin had similar growth rates, whereas supplementation with leukemia inhibitory factor (LIF) resulted in significantly slower growth. However, when OB-cNPC cultures were grown on PDL-fibronectin or PDL supplemented with LIF, a greater proportion of cells with neuronal markers were generated upon differentiation.

Keywords

Canine; olfactory bulb; neural progenitor cells; fibronectin; leukemia inhibitory factor; cell culture

Introduction

Neural stem cells (NSCs) have been isolated from the adult rodent brain by selective response to epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) (Reynolds and Weiss, 1992; Richards et al., 1992; Ray et al., 1993; Palmer et al., 1997). True NSCs are

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multipotential and self-renewing, whereas neural progenitor cells have more restricted renewal and differentiation ability (Seaberg and van der Kooy, 2003). The term 'neural progenitor cell' (NPC) will be used here to include both neural stem and progenitor cells. Neural progenitor cells are excellent candidates for cellular transplantation therapy because they have been shown to replace dead or dying neural tissue, elaborate trophic factors to rescue dysfunctional endogenous neurons, inhibit inflammation, and deliver therapeutic proteins in a widely disseminated manner (Snyder et al., 1995; Lacorazza et al., 1996; Ourednik et al., 2002; Einstein et al., 2003; Pluchino et al., 2003).

Olfactory bulb-derived NPCs are attractive as a source of NPCs for autologous transplantation after ex vivo gene correction because the olfactory bulbs are more easily accessed surgically than the subventricular zone (SVZ) or hippocampus. Cells with the stem cell properties of self-renewal and multipotency have been isolated from postnatal murine olfactory bulbs (Gritti et al., 2002; Liu and Martin, 2003). Similarly, neural stem cells isolated from adult human olfactory bulb biopsies contained self-renewing, multipotential neural stem cells as evidenced by clonal analysis (Pagano et al., 2000). Many neurologic diseases that may potentially benefit from NPC transplantation occur in the dog (Griffiths et al., 1981; Haskins et al., 1984; Alroy et al., 1985; Koppang, 1988; Fischer et al., 1998; Wenger et al., 1999). We recently found that defects in canine NPCs occur in a model of a lysosomal storage disease (Walton and Wolfe, 2007). With a brain that is much closer in size and architecture to the human brain, the dog represents a relevant model for NPC transplantation. Moreover, surgical approaches to the olfactory bulb of dogs have been developed, thus there is potential to perform ex vivo gene therapy experiments using autologous transplantation (Bagley et al., 1997; Glass et al., 2000).

Protocols used for human and rodent in vitro NPC cultures differ with respect to concentrations and combinations of growth factors and substrate (Reynolds and Weiss, 1992; Richards et al., 1992; Kilpatrick and Bartlett, 1993; Ray et al., 1993; Gage et al., 1995; Palmer et al., 1997; Moyer et al., 1997; Carpenter et al., 1999; Laywell et al., 2000). NPC response to growth factors can vary with developmental stage, the factors used, and their concentration (Whittemore et al., 1999; Zhu et al., 1999). For example, although NPCs grown in either EGF or bFGF with heparin are multipotential, studies show a preferential gliogenic effect from EGF-derived cultures and an increased capacity for neuronal differentiation in cultures derived from bFGF and heparin, (Whittemore et al., 1999; Zhu et al., 1999). Combining EGF with bFGF and heparin produces a synergistic effect on NPC cultures (Caldwell and Svendsen, 1998; Vescovi et al., 1999; Gritti et al., 1999; Caldwell et al., 2004). Supplementation of NPC medium with leukemia inhibitory factor (LIF) has been found to increase human embryonic NPC proliferation rate and longevity, and enhance ex vivo expansion (Carpenter et al., 1999; Wright et al., 2006). Culture conditions can also affect cell migration. Epidermal growth factor generated NPCs migrate further on a poly-D-lysine substrate (PDL) than bFGF generated NPCs (Zhu et al., 1999). Similarly, the substrate on which NPCs are grown can also affect cell growth and some functions; fibronectin and laminin enhance neurosphere motility and migration, whereas chondroitin sulfate proteoglycan inhibits sphere migration and velocity (Kearns et al., 2003). Lastly, ex vivo expansion of NPCs via epigenetic stimulation appears to differ between species. Human NPC cultures require both EGF and bFGF, and LIF enhances ex vivo expansion, whereas in rodents this is not the case (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Carpenter et al., 1999; Tropepe et al., 1999; Vescovi et al., 1999).

In light of these findings, optimal growth conditions must be determined for NPCs according to developmental stage, species of origin, and intended use. Since treatment for most CNS diseases will be done postnatally, we were interested in modeling conditions that would occur in ex vivo gene therapy using autologous cells. To optimize culture conditions for ex vivo

canine NPC expansion, we evaluated variables that have been shown to maintain neural progenitor cell qualities and/or increase NPC proliferation and expansion in vitro.

Materials and Methods

Experimental animals

Mixed breed dogs were raised in the Animal Models Core of the W. F. Goodman Center for Comparative Medical Genetics, University of Pennsylvania School of Veterinary Medicine. All dogs were treated in accordance with NIH and USDA guidelines for the use of animals in research and all experimental procedures involving dogs were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Isolation of canine olfactory bulb neural progenitor cells (OB-cNPCs)

Three dogs were humanely euthanized at three weeks of age (19–21 days), by intravenous injection of a barbiturate solution. The brain was removed, placed into a balanced salt solution, and then dissected grossly. The olfactory bulbs were separated from the brain, minced and then digested in 0.25% trypsin (Worthington) in a 37°C water bath for 45 minutes to 1 hour. The enzymatic digestion was stopped with addition of fetal bovine serum (FBS; Hyclone). The tissue was then incubated with DNase I (Sigma) for fifteen minutes in a 37°C water bath and triturated to a single cell suspension with successively smaller diameter pipettes, ending at a flame-polished Pasteur pipette. The cell suspension was centrifuged at 700 rpm at 4°C for 8 minutes, resuspended in 10% FBS plating medium (see below), and triturated. The total number of viable cells was determined by manual count on a hemacytometer; cell viability was assessed using trypan blue exclusion (0.4%; Sigma).

Canine OB-NPC culture and expansion

The OB-cNPCs were plated at a concentration of $4 \times 10^4/\text{cm}^2$ in 10% serum-containing plating medium consisting of DMEM:F12 (1:1 ratio; GibcoBRL) supplemented with 10% FBS, 1% N2 supplement (GibcoBRL), 1% antibiotic-antimycotic (PSF)(100 U/mL penicillin; 100 µg/mL streptomycin; 0.25 µg/mL amphotericin B; GibcoBRL), and 1% L-glutamine (2mM; GibcoBRL). After 24–48 hours, the medium was changed to a serum-free feeding medium consisting of DMEM:F12 supplemented with 1% N2 supplement, 1% PSF, and 1% L-glutamine. The standard combination of growth factors consisted of 20 ng/mL epidermal growth factor (EGF) (recombinant murine; Roche), 20 ng/mL basic fibroblast growth factor (bFGF) (recombinant human; Promega), and heparin (5 µg/mL; Sigma). For a subset of cultures, 10 ng/mL leukemia inhibitory factor (LIF) (recombinant human; Chemicon) was added along with the standard growth factor combination. The cNPC cultures were maintained at 37°C in humidified 5% CO₂ tissue culture incubators. Cultures were fed every 3–5 days by changing half of the medium and adding fresh growth factors. Canine NPCs were plated into 25 cm² tissue culture flasks (Corning) coated with 10 µg/mL poly-D-lysine (Sigma) or poly-D-lysine with 5 µg/mL fibronectin (0.1% bovine; Sigma). Cultures were passaged at approximately 90% confluence by trypsinizing (0.05% trypsin-EDTA; GibcoBRL) and replating at a concentration of $4 \times 10^4/\text{cm}^2$.

Neurosphere assay

For neurosphere assays, canine olfactory bulb-derived neurospheres were collected (passage 1), centrifuged at 700 rpm at 4°C for 5 minutes, trypsinized (0.05% trypsin-EDTA; GibcoBRL) at 37°C for 5 minutes, and triturated to a single cell suspension. The suspension was serially diluted to a final concentration of 0.04 cells/µl; 100 µl of OB-cNPC suspension was plated into each well of a 96-well plate (Corning) with 100 µl of filtered, conditioned OB-cNPC growth medium.

Immunocytochemistry

Cultures were differentiated by plating in the absence of growth factors in an 8-well chamber slide (Lab-Tek II; Nalge Nunc) at a concentration of 4×10^4 cells/well in feeding medium supplemented with 1% FBS. Undifferentiated OB-cNPCs were plated at a concentration of 2×10^4 /well onto poly-D-lysine-coated 8-well glass slides (Cel-Line; Erie Scientific) and allowed to attach overnight in feeding medium containing growth factors. Differentiated and undifferentiated cNPC cultures were processed for immunocytochemistry after 10–12 days and 24 hours, respectively. In some cases, retinoic acid (1 μ M; Sigma) or platelet-derived growth factor (10 ng/mL; Sigma) was added to feeding medium in the absence of growth factors to promote differentiation.

The polyclonal primary antibodies used were rabbit polyclonal anti-nestin, 1:60 dilution (rabbit 130; kind gift of R. McKay, NIH) and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), 1:100 dilution (Chemicon). Primary monoclonal antibodies consisted of rat anti-GFAP, 1:1 dilution (IgG; kind gift of V. Lee); mouse anti- β -tubulin III, 1:300 dilution (IgG; Chemicon); mouse anti-MAP2ab, 1:300 dilution (IgG; Chemicon); mouse anti-O4, 1:3 dilution (IgM); and mouse anti-galactocerebroside, 1:1 dilution (IgG3) (both oligodendrocyte markers were generous gifts of J. Grinspan). Secondary fluorescent antibodies used were goat anti-mouse IgG/IgM FITC, 1:300 dilution (Chemicon); goat anti-rabbit IgG Alexa fluor 594, 1:300 dilution (Molecular Probes); goat anti-rabbit IgG Alexa fluor 488, 1:300 dilution (Molecular Probes); and goat anti-mouse IgM Alexa fluor 488, 1:300 dilution (Molecular Probes).

For all intracellular markers, cells were rinsed in Tris-buffered saline (TBS) (50 mM Tris-base, 0.15M NaCl; pH 7.6), fixed for 10 minutes in 4% paraformaldehyde (Sigma), rinsed three times with TBS, blocked in 5% goat serum (GibcoBRL) with 0.1% Triton X-100 (Sigma) for 40 minutes, and then incubated with primary antibody in 1% goat serum with 0.02% Triton X-100 for 1 hour at room temperature or overnight at 4°C. After three TBS washes, the secondary antibody was applied for 1 hour at room temperature or overnight at 4°C. Cell surface marker staining was performed on live cells. The cells were rinsed in TBS, incubated with primary antibody diluted in TBS for 30 minutes, rinsed briefly with TBS, incubated with secondary antibody for 40 minutes, rinsed with TBS, and then fixed for 10 minutes in 4% paraformaldehyde. All slides were washed three times with TBS before mounting in Vectashield containing 4',6 diamidino-2-phenylindole (DAPI; Vector Laboratories).

Quantitation of immunofluorescence

A minimum of 10 fields for each marker was photographed using a SPOT RT camera (Diagnostic Instruments). An effort was made to sample representatively; when differentiation was not uniformly distributed, similar numbers of fields without positive staining were analyzed. The DAPI-stained nucleus was used to count the total number of cells in all fields using a manual tag with Image-Pro Plus software (version 4.0; Media Cybernetics). The percentage of cells that stained positively for an immunofluorescent marker was obtained by averaging the percentage of positive cells for all fields.

Canine OB-NPC population parameters and statistics

For each cell culture, the number of cells plated at the beginning and harvested at the end of each passage was recorded. For comparison of growth on fibronectin and in the presence of leukemia inhibitory factor, OB-cNPCs from three dogs were evaluated in duplicate for each condition. The total potential cell number generated was calculated by multiplying the total number of viable cells harvested after each passage by the ratio of the total cells harvested to the total cells plated. The product for each passage was added to the subsequent passage to produce the total potential cells generated if all cells harvested were plated (Reynolds and

Weiss, 1996). Cell doubling time was determined from the standard formula: doubling time = $[(\log P - \log H)/\log 2]/D$; where P = the number of cells plated, H = the number of cells harvested, and D = the days between plating and harvest (Heuer et al., 2001).

Statistical analysis was performed to determine whether there was a difference in population doubling times and differentiation ability between OB-cNPCs grown under three separate culture conditions. Comparisons of variable culture conditions were performed using the Kruskal-Wallis non-parametric analysis of variance and, where appropriate, the Dunn's multiple comparisons test.

Results

The canine olfactory bulb is a source of multipotential neural progenitor cells (NPCs) that can be expanded in vitro

We isolated a population of cells from postnatal canine olfactory bulbs that could be propagated in serum-free medium containing bFGF/heparin and EGF. Culture conditions selected for NPCs, as evidenced by both the morphology and immunophenotype of the proliferating cells. The vast majority of the population was immunophenotypically and morphologically immature; most cells were nestin-positive and GFAP-positive, with a bipolar, spindled morphology, as reported recently (Walton and Wolfe, 2007). The O4-negative, GalC-negative, GFAP-positive immunophenotype of proliferating canine olfactory bulb NPCs (OB-cNPCs) is inconsistent with the immunophenotype reported for rat and canine olfactory ensheathing cells (Franceschini and Barnett, 1996; Ito et al., 2006). Neurospheres could be generated from wells initially seeded with only one to four OB-cNPCs (Figure 1), and were commonly present in high-density cell cultures. When growth factors were removed and serum added to cell cultures, cells possessing phenotype-appropriate morphology and markers for either oligodendrocytes, astrocytes, or neurons appeared (Table 1; Figure 2).

Comparison of OB-cNPC growth parameters under three different culture conditions

To determine the effect of different culture conditions on cNPC cell growth, phenotype, and differentiation, cells from OB-cNPC isolations were subjected to three experimental variables. The OB-cNPCs were plated onto: 1) poly-D-lysine (PDL) and fed with the standard medium; 2) PDL with fibronectin (PDL-fibro) and fed with the standard medium; or 3) PDL and fed with standard medium supplemented with leukemia inhibitory factor (PDL-LIF). Olfactory bulb cNPCs were isolated from three dogs and plated in duplicate for each variable.

Population doubling times and cell expansion were determined over the course of ten passages. Cell expansion represents the calculated number of cells that would have been produced if all cells were plated after each passage (Reynolds and Weiss, 1996). The OB-cNPCs could be expanded at least 500-fold in the time evaluated, representing up to 28 generations (Figure 3). Population doubling times expressed as the mean $\pm$ SEM were: 2.8 ± 0.2 days (PDL); 4.4 ± 0.6 days (PDL-fibro); and 5.0 ± 0.8 days (PDL-LIF). There was no statistical difference between doubling times when standard medium (EGF with bFGF/heparin) was used with either substrate ($p > 0.05$). LIF supplementation significantly lengthened doubling time compared to standard medium on PDL ($p = 0.0153$); no significant difference was found between PDL-fibro and PDL-LIF doubling times. The OB-cNPC cultures supplemented with LIF produced fewer total cells (Figure 3) and the slower growth of LIF cultures was associated with greater difficulty in passaging; only two of the three isolations survived to passage (P) 10. We noted that maintenance and proliferation of OB-cNPC cultures was dependent upon cell density. Culture under low-density conditions could only be maintained by supplementation with conditioned medium. Thus, cultures that did not adequately proliferate shortly after passaging tended to die.

Immunophenotypic and morphologic comparisons of proliferating OB-cNPCs grown under three different culture conditions

In vivo studies of the rodent SVZ have shown that neural stem cells have a much longer doubling time than progenitor cells (Morshead et al., 1994). Thus, a slower doubling time may reflect a more undifferentiated progenitor. We therefore evaluated OB-cNPC phenotype and differentiation potential to determine whether substrate (fibronectin) or addition of LIF significantly affected either. Aliquots of cultures from each experimental variable at P5 and P10 were plated overnight in the presence of growth factors for analysis of the undifferentiated phenotype; immunophenotyping was performed at P5 and P10 to allow sufficient time for effect.

Regardless of experimental variable, the undifferentiated OB-cNPCs were primarily nestin-positive and GFAP-positive (Table 1). Glial fibrillary acidic protein (GFAP) is a marker of both differentiated astrocytes and neural stem cells (Doetsch et al., 1999; Laywell et al., 2000; Messing and Brenner, 2003; Morshead et al., 2003), whereas nestin is used as a marker of neural progenitor cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl et al., 1990). The neural stem cells of the SVZ are reported to be both nestin + and GFAP + (Doetsch et al., 1997). The GFAP + cells in proliferating (undifferentiated) OB-cNPC cultures were divided into two groups based on cellular morphology and staining. Cells with a small nucleus; unipolar, bipolar, or round shape; a high nuclear to cytoplasmic ratio (N:C ratio); and a non-filamentous, diffuse GFAP staining pattern were considered 'immature' or undifferentiated (Bonaguidi et al., 2005). In contrast, cells with a large nucleus and a stellate or flattened, fibroblast-like morphology, low N:C ratio, and filamentous staining pattern were considered 'mature' because these morphologies are consistent with mature type 1 and type 2 astrocytes (Raff et al., 1983; Bonaguidi et al., 2005) (Figure 4).

Since polyclonal anti-GFAP antibodies have been shown to cause spurious GFAP staining, likely due to vimentin or nestin cross-reactivity (Zhang, 2001), we co-stained cNPC cultures with a polyclonal rabbit GFAP antiserum and a rat monoclonal anti-GFAP antibody to verify that GFAP staining was specific. The polyclonal and monoclonal anti-GFAP antibodies stained the same cells. In addition, the same two types of staining patterns in immature and mature cells (diffuse and filamentous, respectively) were observed whether polyclonal or monoclonal antibodies were used (Figure 5). Rabbit anti-human nestin was previously shown to react with a population of multipotential canine NPCs derived from the striatum and ventral mesencephalon (Milward et al., 1997).

While most of the cells in proliferating (undifferentiated) cultures were nestin + and GFAP + / immature, there was a small population that was morphologically and immunophenotypically of the mature type (Table 1). At P5, fewer than 4% of cells were β -tubulin III + or Map2ab + with neuronal morphology and < 20% of cells were GFAP + with mature astrocytic morphology. There also was a small population of Map2ab + cells with an immature, non-neuronal morphology (small unipolar or bipolar cells with a high N:C ratio; Table 2 and Figure 6). Of the three growth conditions, LIF-supplemented OB-cNPC cultures contained significantly more of these Map2ab + / immature cells at P5 ($p < 0.01$) than the other conditions, and only the LIF-supplemented cNPC cultures contained Map2ab + / immature cells at P10.

Immunophenotypic comparison of differentiated OB-cNPCs grown under three different culture conditions

For analysis of the differentiated phenotype, aliquots were plated for 10–12 days in 1% serum-supplemented medium in the absence of growth factors. Removal of growth factors resulted in proportions of cells that stained positively for neuronal, astrocytic, and oligodendrocytic markers, and displayed a phenotype-appropriate morphology (Table 1 and Figure 2). In this

passive differentiation assay, the greatest amount of differentiation was observed at P5. Immunologic and morphologic markers of differentiated neurons, astrocytes and oligodendrocytes were observed regardless of culture conditions. In addition, the proportion of cells that were double-positive for nestin and GFAP, with immature morphology, was generally lower than seen in the growth factor-maintained cultures at the same passage, but the difference did not reach statistical significance in these experiments. In contrast, there were significantly more mature Map2ab + cells present in PDL-fibro and PDL-LIF cultures than were present in PDL cultures ($p < 0.001$). Cultures supplemented with LIF also contained significantly more O4 + cells, a marker for the oligodendrocyte lineage, than PDL-fibro or PDL ($p < 0.001$).

By P10, spontaneous differentiation upon growth factor withdrawal was absent across all experimental variables. Although there were GFAP + cells with mature morphology, the staining pattern was immature, i.e., diffuse rather than filamentous (Figure 4). These GFAP + cells were classified as neither immature progenitor cells nor mature astrocytes, but as an 'intermediate' type of GFAP + cell (Table 1) (Walton and Wolfe, 2007). In order to determine whether the lack of differentiation at P10 was a function of the passive method of differentiation (withdrawal of growth factors), we attempted to potentiate differentiation with retinoic acid or platelet-derived growth factor (PDGF) (Takahashi et al., 1999; Mujtaba et al., 1999), but neither of these compounds increased differentiation in the P10 cultures. This appeared to be due to the inability of the cells to respond to the growth factors used, however, since neither growth factor increased the number of differentiated cells in cNPC cultures at P1 (data not shown data).

Discussion

The dog has a number of features that make it an excellent model for NPC transplantation: 1) the brain is much closer in size and more similar in physical organization to the human brain than the brains of rodents; 2) there are many well-defined neurological diseases, including genetic diseases, that are essentially the same as in human patients; and 3) relatively large numbers of offspring can be produced. Moreover, if neural progenitor cells (NPCs) could be obtained and adequately expanded from a surgically accessible site such as the olfactory bulb, the dog would serve as a valuable large animal model for autologous transplantation. However, such experiments may involve increased expense, specific requirements for USDA-covered species, and specialized facilities.

Leukemia inhibitory factor (LIF) has been shown to aid in the maintenance of neural stem cells, both in vivo and in vitro (Carpenter et al., 1999; Shimazaki et al., 2001; Pitman et al., 2004; Bonaguidi et al., 2005). In mice, LIF increases the proportion of GFAP + immature NPCs within days of administration (Bonaguidi et al., 2005), whereas the effects of LIF on human fetal NPC growth are not apparent until 50–60 days in culture (Carpenter et al., 1999). The canine olfactory bulb NPCs (OB-cNPCs) were maintained in LIF-supplemented medium for ten passages (79–80 days), which should be sufficient time even for a delayed positive effect to have been seen. Our data showed that the OB-cNPC cultures supplemented with LIF had a significantly slower doubling time and generated fewer cells than cNPCs grown without LIF. For autologous transplantation purposes, the growth delay and fewer total cells generated in LIF-supplemented cultures might not be considered desirable. On the other hand, if the slower doubling time reflects a less mature phenotype, having a higher proportion of progenitor cells in the transplanted population may be advantageous in some applications, particularly during development.

The LIF-supplemented OB-cNPC cultures contained significantly more Map2ab + /immature cells at passage 5 (P5) among the undifferentiated cultures in the three growth conditions. In addition, only the LIF-supplemented cNPC cultures contained Map2ab + immature cells at

P10. While there was a small proportion of Map2ab + cells with mature neuronal morphology that represented differentiated cells, most of the Map2ab + cells had an immature morphology. It has previously been shown that subsequent to CNS injury, reactive astrocytes can express the neuronal antigens *Tau*, Map2, neuron-specific enolase, and GABA, in addition to GFAP and nestin (Lin and Matesic, 1994; Frisen et al., 1995; Schinstine and Iacovitti, 1996; Sahin Kaya et al., 1999). It has been hypothesized that reactive astrocytes represent either astrocytes that revert to bipotential progenitors (cells that show both neuronal and glial phenotypes) or that reactive astrocytes are recruited from neural stem cells (Lin et al., 1995; Schinstine and Iacovitti, 1996). Evidence to support the latter comes from both in vitro and in vivo studies. In vitro, primary astrocytes derived from mouse brain were nestin + and GFAP +, but showed no immunoreactivity to the neuronal antigens Map2 and *Tau*. However, astrocytes derived from neurospheres maintained in EGF, were positive for GFAP, nestin, Map2 and *Tau* (Schinstine and Iacovitti, 1996). In mice, cortical injury studies suggest that nestin + cells migrate from the subventricular zone to the site of injury (Frisen et al., 1995; Lin et al., 1995). Thus, the Map2ab + cells with immature morphology in the OB-cNPC cultures may represent a cell type analogous to reactive astrocytes. Supplementation with LIF appears to enhance this population.

When growth factors were removed in OB-cNPC cultures to induce differentiation, LIF-supplemented cultures did generate significantly more mature Map2ab + and O4 + cells compared to standard culture conditions. In human fetal cortical NPC cultures, LIF supplementation is reported to generate significantly more β -tubulin III + cells than in EGF-maintained cultures (Wright et al., 2006), and addition of LIF to adult human olfactory bulb cultures enhances neuronal potential (Pagano et al., 2000). In rodents, LIF and LIF-related cytokines signaling via the LIF receptor increase oligodendrocytic generation and survival (Mayer et al., 1994; Barres et al., 1996). In the dog cells, these results were observed only up to P5. By P10, LIF cultures, as all other cultures, did not generate mature neurons or glial cells. It seems unlikely that the decreased staining for mature markers is due to a lack of progenitors because one would expect that the undifferentiated cultures would then be composed largely of mature, differentiated cells, which is not the case. It is possible that after prolonged growth factor exposure, the withdrawal of growth factors and exposure to low serum concentration were insufficient stimuli for differentiation. However, the cells did not have an enhanced differentiation response to retinoic acid or PDGF at either early or late passage cultures. Although retinoic acid has been reliable for differentiation of primary mouse NSCs in our hands (Heuer et al., 2001), it is possible that other factors might prove more effective to induce differentiation in OB-cNPCs, for example neurotrophins or higher concentrations of fetal bovine serum. Similarly, it is possible that late passage cells might differentiate if they were transplanted into a neurogenic region in vivo, since more complex differentiation cues may be present.

Fibronectin is an important part of the extracellular matrix and is present in the ventricular and subventricular zones of postnatal rats and mice (Hynds and Snow, 2001; Campos et al., 2004). When used to facilitate NPC retroviral transduction, it increases NPC proliferation in the neonatal mouse (Rappa et al., 2004). In contrast, in the dog we found that fibronectin did not enhance growth rate or expansion of OB-cNPCs. Since the neonatal dog brain is developmentally more advanced than a neonatal rodent brain (Clancy et al., 2001), a better comparison may be to the adult rodent brain. The growth rate of adult rat SVZ-derived NPCs has been compared using various combinations of substrates and growth factors (Whittemore et al., 1999). Similar to our findings for canine OB-NPCs, adult rat NPCs grown on polyornithine-fibronectin with bFGF and heparin did not proliferate more than the other substrates used.

While LIF-supplemented OB-cNPC cultures demonstrated slower expansion potential, there was enhanced differentiation into neuronal and oligodendrocytic lineages. More cells with a neuronal phenotype were also produced when fibronectin was included as a substrate. These findings support the use of both substances in ex vivo propagation to promote neuronal potential in transplants. Transplantation studies into neurogenic regions of the dog brain will be necessary to determine if these results are recapitulated in vivo. Characterization of postnatal canine olfactory bulb NPCs should also aid in the development of an autologous transplantation model for neurodegenerative disease in a brain that is scaled to that of a human.

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Figure 1. Neurosphere generation (96-well culture plate). (A) Single well, day 6 in vitro; 6 cells were detected. By day 18 in vitro, over 100 cells were present and neurospheres were noted in other wells (B). Bar = 50µm (A); 100µm (B).

Figure 2.

Immunophenotype of canine olfactory bulb NPCs (OB-cNPCs) after growth factor withdrawal and addition of 1% serum. (A) β -tubulin III + cells with neuronal morphology (arrowhead); (B) GFAP + cells with filamentous staining and mature astrocytic morphology; (C) O4 + cells with oligodendrocyte morphology. Bar = 20 μ m (A, C); 40 μ m (B).

Figure 3.

Cell numbers represent the calculated number of total OB-cNPCs produced after nine passages (starting with 1×10^6 cells) if all the cells generated had been plated. After P8, the data for PDL-LIF was based on duplicate cultures from the surviving two isolations. PDL, poly-D-lysine substrate with standard growth factors; PDL-fibro, poly-D-lysine plus fibronectin substrate with standard growth factors; PDL-LIF, poly-D-lysine substrate with standard growth factors and leukemia inhibitory factor (LIF).

Figure 4.

GFAP + cells showed three types of morphology and staining. (A) The majority of cells grown in the presence of growth factors were small, round or bipolar, and stained diffusely for GFAP. (B) Both immature (arrow) and mature (arrowhead) GFAP + cells were present in differentiation cultures. Mature GFAP + cells have a filamentous staining pattern and multiple processes. (C) At passage 10, after growth factor withdrawal the GFAP + cells had a diffuse, disorganized staining pattern (immature pattern), but a flattened, epithelioid morphology (mature morphology). (D, E) Examples of filamentous (D) and diffuse, disorganized (E) staining are shown. Bar = 25 μ m (A, C); 50 μ m (B); 12.5 μ m (D, E).

Figure 5. Anti-GFAP staining of canine OB-NPCs using polyclonal and monoclonal antibodies. (A) Rabbit polyclonal anti-GFAP with a secondary Alexa fluor 594 antibody (red). (B) Rat monoclonal anti-GFAP with a FITC secondary antibody. (C) Merged image showing co-staining of GFAP + cells with both primary antibody types. Note the presence of two types of GFAP staining: diffuse and filamentous. Bar = 25 μ m.

Figure 6. Morphology of Map2ab + cells in OB-cNPC cultures. (A) In the presence of growth factors, small Map2ab + cells are noted with bipolar or unipolar morphology. (B) Upon differentiation by withdrawal of growth factors and addition of serum, variably sized cells with multiple processes are observed (arrowheads). Bar = 20 μ m.

Table 1
Undifferentiated and Differentiated Immunophenotypes of Canine Olfactory Bulb NPCs

Passage	Culture Conditions ^a		Culture Type	Progenitor Cell		Intermediate		Astrocytic		Mature Neuronal		Oligodendrocytic	
	PDL	PDL-fibro	PDL-LIF	Nestin	GFAP, small uni-/bipolar; high N:C ratio; diffuse stain	GFAP, large round/flat; low N:C ratio; diffuse stain	GFAP, large stellate; low N:C ratio; filamentous stain	β -Tubulin	Map2ab	O4	GalC		
5	+	-	-	95.5 $\pm$ 1.3	79.1 $\pm$ 4.7	0.0	16.6 $\pm$ 4.3	0.0	1.6 $\pm$ 1.6	0.0	0.0		
	+	-	-	84.6 $\pm$ 2.9	48.0 $\pm$ 7.0	0.0	31.0 $\pm$ 5.9	14.2 $\pm$ 3.1	8.2 $\pm$ 3.0	2.6 $\pm$ 1.5	0.0		
	+	+	-	98.1 $\pm$ 0.5	86.3 $\pm$ 2.5	0.0	6.6 $\pm$ 1.2	3.9 $\pm$ 2.5	0.3 $\pm$ 0.2	0.0	0.0		
	+	+	-	92.4 $\pm$ 1.3	24.1 $\pm$ 5.6	0.0	17.8 $\pm$ 3.0	7.7 $\pm$ 1.8	14.6 $\pm$ 2.4	3.4 $\pm$ 1.1	5.9 $\pm$ 3.2		
10	+	-	+	94.3 $\pm$ 1.4	67.9 $\pm$ 4.7	0.0	18.5 $\pm$ 2.7	3.1 $\pm$ 0.7	3.8 $\pm$ 1.1	0.0	0.0		
	+	-	+	83.2 $\pm$ 4.3	41.8 $\pm$ 8.0	0.0	40.6 $\pm$ 6.7	13.2 $\pm$ 2.7	16.5 $\pm$ 2.7	6.8 $\pm$ 2.3	0.8 $\pm$ 0.3		
	+	-	-	92.8 $\pm$ 1.5	92.5 $\pm$ 2.3	1.5 $\pm$ 0.7	0.0	0.0	0.0	0.0	0.0		
	+	-	-	91.6 $\pm$ 2.5	48.4 $\pm$ 4.9	43.5 $\pm$ 6.3	0.0	0.0	0.0	0.0	0.0		
	+	+	-	97.7 $\pm$ 0.5	99.0 $\pm$ 0.6	0.6 $\pm$ 0.6	0.0	0.0	0.0	0.0	0.0		
	+	+	-	95.6 $\pm$ 1.3	87.1 $\pm$ 2.1	12.9 $\pm$ 2.1	0.0	0.0	0.0	0.0	0.0		
	+	-	+	96.5 $\pm$ 0.9	93.7 $\pm$ 2.5	0.0	0.0	0.0	0.0	0.0	0.0		
	+	-	+	96.7 $\pm$ 1.0	73.1 $\pm$ 6.0	28.4 $\pm$ 5.5	0.0	0.0	0.0	0.0	0.0		

For each experimental variable, values represent the mean $\pm$ SEM for duplicate cultures from three isolations.

^a PDL, poly-D-lysine substrate with standard growth factors; PDL-fibro, poly-D-lysine plus fibronectin substrate with standard growth factors; PDL-LIF, poly-D-lysine substrate with standard growth factors and leukemia inhibitory factor (LIF).

^b Growth, proliferation culture;

^c Diff, differentiation culture.

Table 2

Morphology of Map2ab + Cells in Undifferentiated Canine Olfactory Bulb NPC Cultures

Passage	Growth Condition	Map2ab Immunopositivity (%)	
		Mature neuronal morphology	Immature small, uni-/bipolar; high N:C ratio
5	<i>PDL</i> ^a	1.6 ± 1.6	5.0 ± 1.9
	<i>PDL-fibro</i> ^b	0.3 ± 0.2	5.7 ± 1.8
	<i>PDL-LIF</i> ^c	3.8 ± 1.1	15.9 ± 3.0
10	<i>PDL</i>	0.0	0.0
	<i>PDL-fibro</i>	0.0	0.0
	<i>PDL-LIF</i>	0.0	11.6 ± 3.7 [†]

For each experimental variable, values represent the mean ± SEM for duplicate cultures from three isolations.

^aPDL, poly-D-lysine substrate with standard growth factors;

^bPDL-fibro, poly-D-lysine plus fibronectin substrate with standard growth factors;

^cLIF, poly-D-lysine substrate with standard growth factors and leukemia inhibitory factor (LIF).

[†]Value is the mean ± SEM from duplicate cultures of the two isolations that survived.