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Colony-forming progenitors from mouse olfactory epithelium: Evidence for feedback regulation of neuron production

(stem cell/olfactory receptor neuron/neurogenesis/neural precursor/cell interactions)

JEFFREY S. MUMM*, JIANYONG SHOU*, AND ANNE L. CALOF†

Department of Anatomy and Neurobiology and Developmental Biology Center, University of California, Irvine, College of Medicine, Irvine, CA 92697-1275

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ABSTRACT **The mammalian olfactory epithelium (OE) supports continual neurogenesis throughout life, suggesting that a neuronal stem cell exists in this system. In tissue culture, however, the capacity of the OE for neurogenesis ceases after a few days. In an attempt to identify conditions that support the survival of neuronal stem cells, a population of neuronal progenitors was isolated from embryonic mouse OE and cultured in defined serum-free medium. The vast majority of cells rapidly gave rise to neurons, which died shortly thereafter. However, when purified progenitors were co-cultured with cells derived from the stroma underlying the OE, a small subpopulation (0.07–0.1%) gave rise to proliferative colonies. A morphologically identifiable subset of these colonies generated new neurons as late as 7 days *in vitro*. Interestingly, development of these neuronal colonies was specifically inhibited when purified progenitors were plated onto stromal feeder cells in the presence of a large excess of differentiated OE neurons. These results indicate that a rare cell type, with the potential to undergo prolonged neurogenesis, can be isolated from mammalian OE and that stroma-derived factors are important in supporting neurogenesis by this cell. The data further suggest that differentiated neurons provide a signal that feeds back to inhibit production of new neurons by their own progenitors.**

The cellular steps and molecular mechanisms underlying the generation of neurons in higher vertebrates are relatively poorly understood. In particular, the question of whether most neuronal lineages derive from true stem cells—self-renewing cells with the capacity to sustain continual neurogenesis—has been difficult to resolve. Although there is mounting evidence demonstrating the existence of multi-potent progenitor cells in the developing nervous system, the fact that most regions of the nervous system are not inherently self-renewing (i.e., do not regenerate their neurons following neuronal death) suggests that most neuronal lineages may operate without stem cells, instead generating a complement of post-mitotic neurons and leaving behind no proliferating cells capable of recapitulating the process (reviewed in ref. 1).

The vertebrate olfactory epithelium (OE) has long been thought to harbor a neuronal stem cell because generation of olfactory receptor neurons (ORNs) takes place continuously in the OE, from embryogenesis throughout adult life (2). Although the OE was perceived initially as a relatively simple neuroepithelium, identification of the stem cell in this tissue has proved elusive. Earlier studies of neurogenesis and neuronal regeneration *in vivo* suggested the basal cells of the OE as candidates for stem cells (3–5), but more recent work indicates that only a subset of these (the so-called globose basal cells) are actually in the neuronal lineage (6, 7). Furthermore, *in vitro* and *in vivo* studies indicate that globose basal cells themselves are a heterogenous population. The majority of these cells—called "Immediate Neuronal Precursors" or INPs *in vitro*—are transit amplifying cells rather than stem cells (i.e., they are committed precursors that go through several symmetrical divisions followed by differentiation of all progeny into neurons) (6, 8, 9). In addition, new studies have shown that there are at least two stages of neuronal transit amplifying cells interposed between the stem cell and the post-mitotic ORN in the ORN lineage: Mammalian achaete-scute homolog-1 expression demarcates cells at the earliest transit amplifying stage so far identified; this cell stage appears to be interposed between the stem cell and the INP (10).

In this study, we develop methods to purify neuronal progenitor cells from OE and characterize their development *in vitro*. The basic procedure employed is a negative selection in which immunological panning is used to selectively remove post-mitotic ORNs from a starting population of cells consisting of ORNs and their progenitors; the resulting population consists of >96% pure neuronal progenitors. We take advantage of differential survival of purified progenitors to identify neuronal colony-forming cells (CFUs, colony-forming units) that are able to continuously generate ORNs for up to 7 days *in vitro*. Feeder cells from stroma underlying the OE appear to be crucial to maintain viable neuronal CFUs *in vitro*, but differentiated neurons specifically inhibit formation of neuronal CFUs. These results have important implications for understanding the mechanisms by which neuron production is regulated in the developing and regenerating nervous system.

MATERIALS AND METHODS

Animals. CD-1 (Charles River Breeding Laboratories) and transgenic ROSA26 (TgR[ROSA26]26Sor, The Jackson Laboratory) mice were naturally mated. Vaginal plug detection established day 0.5 of pregnancy. Tissue for all experiments was taken from embryonic day 13.5–15.5 embryos.

Materials. Media, antibiotics, and merosin (MN, human) were from GIBCO/BRL. ^{3}H-TdR (70–90 Ci/mmol); 1 Ci = 37 Gbq) was from New England Nuclear. NTB2 emulsion, D-19 developer, and fixer were from Eastman Kodak. Glass coverslips (12 mm, no. 1 thickness) were from Propper Manufacturing (Long Island City, NY). Clinical reagent grade and crystallized bovine serum albumin (CRG-BSA and BSA) were from ICN. Bovine calf serum (A-2151-L) and serum-free hybridoma medium (HyQ-CCM™1) were from HyClone. Unless noted, all other reagents were from Sigma.

Tissue Culture. For purified progenitor cell cultures, OE was isolated from embryonic nasal turbinates (6) cultured in suspension for 8–10 hr, and the neuronal cell fraction (ORNs plus neuronal progenitors) prepared as described (11), except that dispase (50 μg/ml; Boehringer Mannheim Grade I, >6 units/mg) was used instead of trypsin for cell dissociation. After panning

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Abbreviations: OE, olfactory epithelium; ORN, olfactory receptor neuron; CFU, colony-forming unit; NCAM, neural cell adhesion molecule; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; β-gal, β-galactosidase.

*J.S.M. and J.S. contributed equally to this work.
†To whom reprint requests should be addressed at: Department of Anatomy and Neurobiology, 364 Med Surge II, University of California, Irvine, College of Medicine, Irvine, CA 92697-1275. e-mail: alcalof@uci.edu.

(see below), purified progenitors were resuspended in low calcium culture medium containing 5 mg/ml crystalline BSA (LCM) (12) and plated onto stroma cell feeder layers.

Stromal cells for feeder layers were isolated from CD-1 turbinate tissue remaining following OE purification. Stroma was dissociated by trituration, filtered through 20-μm nylon mesh (Small Parts) to remove aggregates, and plated in DMEM with 10% bovine calf serum. Cells were passaged 1–4 times prior to use. To generate feeder layers, cells were treated for 2 hr with mitomycin C (10 μg/ml), rinsed thoroughly in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS), and plated overnight at a density of 4×10^4 cells per well in 96-well tissue culture trays (Costar, 3596). Approximately 1 hr prior to plating purified progenitors onto them, feeder layers were rinsed in CMF-HBSS and switched into LCM.

Immunological Purification of Progenitors. Serum-free culture supernatant (200 ml) from H28 rat anti-NCAM (neural cell adhesion molecule) hybridoma cells (9, 13) was precipitated with 50% saturating ammonium sulfate at 4°C overnight, pelleted by centrifugation, and the undialyzed precipitate diluted to 30 ml in CMF-HBSS and stored at −80°C (14). Panning plates were prepared by coating 100-mm Petri dishes (Fisher, 08-757-13) sequentially with goat anti-rat IgG (Cappel, 55722, 50 μg/ml) in 50 mM Tris buffer (pH 9.5) followed by 5 ml of H28 precipitate, each overnight at 4°C. One hour before use, panning plates were rinsed extensively with CMF-HBSS and blocked in CMF-HBSS containing 10 mg/ml CRG-BSA.

OE neuronal cell fractions were resuspended in 5 ml of panning medium: L15 supplemented with BSA (1 mg/ml), glucose (3 mg/ml), insulin (10 ng/ml), transferrin (10 μg/ml), progesterone (20 nM), putrescine (100 μM), selenium (300 nM), and penicillin–streptomycin (40 units/ml). Cell suspensions were incubated on the panning plate for 30 min at room temperature in the dark, with intermittent agitation. Cells remaining in suspension were collected, centrifuged for 5 min at $100 \times g$, resuspended in LCM, and plated at indicated densities. To assess purity, cells were removed from suspension, before and after panning, plated onto poly-D-lysine (1 mg/ml in water)-coated coverslips, incubated for 30 min at 4°C, then fixed with Omnifix II (AnCon Genetics) for 10 min and analyzed for NCAM immunoreactivity using an independent anti-NCAM IgG (AG1D5) (6). Pre-pan suspensions consisted of 42.8% (±2.0 SEM, $n = 10$ experiments) $NCAM^-$ cells. Post-pan suspensions achieved a 96.1% (±0.4 SEM, $n = 10$ experiments) purification of the $NCAM^-$ population. OE purified from a single embryo yielded on the order of $0.5–0.8 \times 10^5$ neuronal cells; yield of purified progenitors was ≈20% of total cells panned, typically.

Immunocytochemistry and β-Galactosidase (β-gal) Detection. For detection of NCAM expression in cultured cells, cultures were fixed for 10 min in 3.7% formaldehyde/5% sucrose in calcium- and magnesium-free Dulbecco's PBS, incubated with undiluted H28 rat anti-NCAM hybridoma supernatant, and detected as described (9). For β-gal histochemistry, cultures were fixed for 15 min in 0.5% glutaraldehyde/5% sucrose/2 mM $MgCl_2$ in PBS (pH 7.5), then stained with 0.1% Triton/0.01% deoxycholic acid/2 mM $MgCl_2$/5 mM $K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$ in PBS (pH 7.5) containing 320 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

RESULTS

In previous studies, a method was developed for isolating a neuronal cell fraction from the mouse OE (11, 12). Briefly, pieces of purified embryonic mouse OE are cultured in suspension, during which time neuronal cells (ORNs and neuronal progenitors) sort out from other cells present in the epithelium and may be removed enzymatically. Since it has previously been shown that NCAM is expressed only by post-mitotic ORNs, but not by their progenitors (6), we modified this method further, to isolate a population highly enriched in neuronal progenitors. This was accomplished by immunological panning, in which anti-NCAM-treated dishes were used to selectively remove post-mitotic ORNs from the neuronal cell fraction. As described in *Materials and Methods*, this procedure yielded a preparation that was >96% NCAM-negative, presumptive ORN progenitors.

When these cells were plated in defined serum-free medium, onto polylysine/merosin coated substrata, most behaved similarly to neuronal progenitors that have been described in explant cultures of embryonic mouse OE (6, 9). Over the course of the first 2 days in culture, nearly all (95.6 + 1.1%) differentiated into NCAM-expressing ORNs (Fig. 1*A*, dashed line). Thus, the cells purified using the immunological panning procedure are identifiable functionally as ORN progenitors. When cultured for longer than 2 days in these conditions, however, the newly-generated ORNs and any remaining undifferentiated progenitors died, leaving essentially no living cells remaining by 7 days *in vitro* (Fig. 1*B*, dashed line). Even the addition of Fibroblast Growth Factor-2, which, in explant cultures, prolongs neurogenesis for up to 4 days (9), failed to halt the disappearance of living cells from these cultures by the end of a week (data not shown). Thus, if a cell capable of sustaining neurogenesis is present in the purified progenitor cell population, it either does not survive, or it does not give rise to neurons, under these conditions.

In an effort to enhance survival and/or proliferation of neuronal progenitors, we turned to co-culturing these cells over monolayers of feeder cells isolated from the stroma underlying the OE. Because proliferative cells in the OE lie relatively close to the basal lamina *in vivo*, it seemed possible that factors derived from subjacent stroma might play a role in supporting neurogenesis. To facilitate identification of OE neuronal progenitors that were cultured together with stromal cells, OE progenitors were purified from ROSA26 mice, which express a *lacZ* transgene in essentially all cells (16). ROSA26-derived cells and their progeny can be identified histochemically, using the β-gal substrate X-gal, or immunocytochemically, using antibodies to bacterial β-gal.

When purified ROSA26-derived progenitors were plated onto monolayers of growth-arrested stromal cells, the majority behaved identically to purified progenitors cultured on polylysine/merosin substrata: they rapidly differentiated into $NCAM^+$ neurons (Fig. 1*A*, solid line). However, in the co-culture condition, numbers of ROSA26-derived cells declined only for about 4 days,

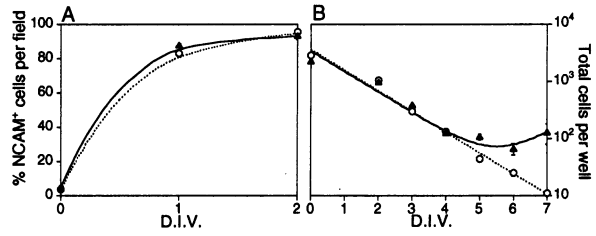

FIG. 1. Differentiation and survival of purified OE progenitor cells. (*A*) The majority of cultured progenitor cells rapidly differentiate into NCAM-expressing ORNs. Approximately 4000 purified ROSA26 progenitors were plated onto 13-mm-diameter glass coverslips coated with polylysine and merosin (9) (dashed line, ○) or monolayers of growth-arrested stroma (solid line, ▲). Cultures were grown for indicated times in LCM, then fixed and double-labeled for NCAM (H28 monoclonal) and β-gal [rabbit anti-β-gal serum, 1:1000 dilution (15)]. The percentage of β-gal-expressing cells that were also $NCAM^+$ was scored for a minimum of 150 cells in 25 randomly-chosen fields for each of 2 coverslips in each condition at each time. Data points show mean ± range. (*B*) A subset of progenitors survives and proliferates when cultured over stroma feeder cells. Purified ROSA26 progenitors were plated onto poly-D-lysine/merosin substrata (dashed lines, ○) or monolayers of CD-1 stroma (solid lines, ▲) in 96-well tissue culture plates. Cultures were fixed and processed for X-gal histochemistry at 4 hr, 2 days, and every day thereafter for 7 days in culture. The total number of $X\text{-gal}^+$ cells was counted in each well. Data points show mean ± SEM for quadruplicate wells at each time.

after which they began to increase again (Fig. 1*B*, solid line). This result was the first indication that a fraction of purified progenitors was capable of sustained proliferation under appropriate culture conditions.

Examination of such cultures at the end of 7 days indicated that most surviving *lacZ*-expressing cells were found in tight clusters containing five or more cells (Fig. 2). Despite the existence of considerable variation in cell morphology among clusters (Fig. 2 *A–D*), cells within a cluster always had a similar morphology, suggesting that clusters were colonies derived from clonal expansion. Indeed, in a number of cases ($n = 11$), clusters were identified by phase-contrast microscopy in living cultures and followed for several days, during which time progressive increases in cluster size were seen (data not shown). These results indicate that, when cultured over stromal cells, a fraction of purified OE progenitors is capable of surviving and giving rise to colonies that expand over time. Fig. 3 presents data on the frequencies with which such colonies arise as a function of the number of cells plated. Fitting the data to a curve based on the zero order term of the Poisson equation (17) indicates that colonies arise from 0.07–0.1% of input cells. This suggests that only very rare cells are capable of giving rise to colonies, at least under these culture conditions.

If colony-forming cells are progenitors of neurons, it seemed possible that they might continue to give rise to neurons *in vitro*. When co-cultures were examined after 7 days, a subset of colonies was found that contained tightly clustered cells, many of which had obvious neurites (Fig. 2*D*). When stained with an antibody to NCAM, this colony type was found to contain NCAM$^+$ ORNs among its cells (Fig. 2*E*); NCAM$^+$ cells were never found in other colony types, nor were they found in cultures of stroma alone. By examining cell morphologies, it was also possible to identify three distinct colony types that did not contain neurons: these consisted of medium-sized (30–50 μm diameter) round cells (Fig. 2*A*); spindle-shaped cells, with a morphology resembling that of olfactory ensheathing or Schwann cells (18, 19) (Fig. 2*B*); or flattened fibroblastic cells (Fig. 2*C*). In contrast, the presence of NCAM$^+$ neurons correlated with colonies that contained small (<10 μm diameter) round cells that typically remained tightly associated with each other (Fig. 2*D*). From comparisons of parallel experiments in which cultures were stained with either X-gal or anti-NCAM, we estimated that about one-fourth (26% ± 3% SEM) of the colonies that appear in these cultures contain neurons.

The existence, at 7 days in culture, of neuron-containing colonies does not prove the existence of neuron-producing colonies. Neurons found in colonies could merely be survivors from the large numbers of neurons that are generated during the first 1–2 days in culture. Given the propensity of ORNs to migrate long distances *in vitro* (11), and the propensity of these cells to adhere to each other, it is not unreasonable to imagine that ORNs could arrive in colonies by accretion, rather than by *de novo* production. To address this question, progenitor/stroma co-cultures were

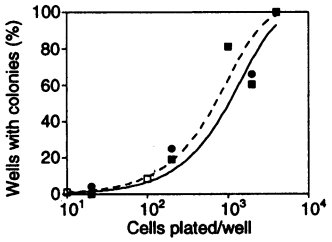

FIG. 3. Frequency of colony-forming cells in purified progenitor preparations. Purified ROSA26 progenitors were plated at indicated densities onto CD-1 stromal cells in 96-well tissue culture plates. Cultures were maintained for 7 days, then fixed, stained with X-gal, and scored for the presence of X-gal$^+$ colonies. Plating efficiency was determined from parallel experiments in which three wells at each density were fixed 6–8 hr after plating and the total X-gal$^+$ cell number counted. Plating efficiency averaged 62% (±8% SEM), and was not significantly different at different plating densities. Wells were scored for the presence or absence of colonies, rather than the number of colonies per well, to avoid systematic errors that might stem from mistaken identification of adjacent colonies as a single colony. The apparent frequency of CFUs was inferred from the zero-order term of the Poisson equation, which relates that frequency to the fraction of wells that contain no colonies (17). The data were fit to the equation $y = 100(1\text{-}e^{-fX})$, where y = the percentage of wells with colonies, f = the frequency of CFUs, and X = the number of cells plated per well. Data from three experiments are plotted (□, ■, ●). Solving the equation for the two data sets with >2 points yields an apparent frequency of CFUs of 1 in 1013 (■, dashed line) or 1 in 1491 (●, solid line) of the total progenitors plated per well.

pulsed with [^{3}H]thymidine for a total of 10 hr at 6 days in culture, then chased with unlabeled thymidine for a further 24 hr (total time in culture ≈7.5 days), fixed, immunostained for NCAM, and processed for autoradiography. As shown in Fig. 4, a substantial number of the NCAM$^+$ cells in neuron-containing colonies were ^{3}H-labeled. Thus, these neurons were generated during days 6–7 in culture, indicating that ORN production was ongoing at a time much later than has so far been seen *in vitro* (see ref. 9). Importantly, NCAM-negative cells were also ^{3}H-labeled in neuronal colonies. This indicates that not all cells in these colonies have differentiated, as expected if colonies contain not only ORNs but also surviving neuronal progenitors.

In the OE *in vivo*, increases in progenitor cell proliferation can be triggered by increased death of post-mitotic ORNs, suggesting either that dying ORNs produce a signal that upregulates neuron progenitor proliferation or that living ORNs produce a signal that inhibits progenitor proliferation (8, 10, 12). To determine if evidence for an inhibitory signal could be obtained *in vitro*, experiments were performed in which purified ROSA26 progenitors were plated onto growth-arrested CD-1 stromal feeder layers in the normal manner. In one-half of the cultures, however,

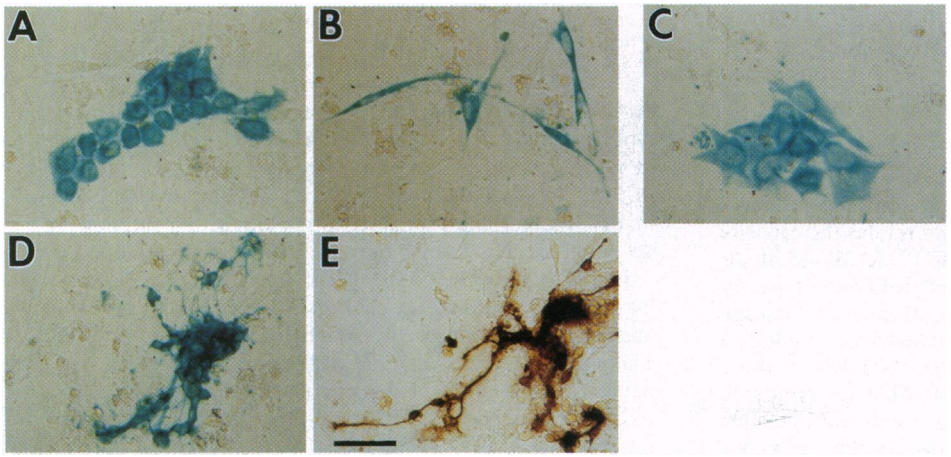

FIG. 2. Co-cultured progenitors generate colonies with four distinct cellular morphologies, one of which is neuronal. Purified ROSA26 progenitors were co-cultured over monolayers of CD-1 stroma in 24-well plates. Cultures were fixed and processed with X-gal (*A–D*, blue) or for NCAM immunoreactivity (*E*, brown) after 7 days *in vitro*. Four distinct colony morphologies are observed: (*A*) medium-round cells; (*B*) spindle-shaped cells; (*C*) polygonal fibroblastic cells; and (*D*) small, round cells, many with neuritic processes. A single colony of the small round cell type is shown stained for NCAM in *E*. Many NCAM$^+$ cells with distinctly neuronal morphology (neurites tipped with growth cones) are observed. This last colony type was subsequently termed "neuronal." (Bar = 50 μm.)

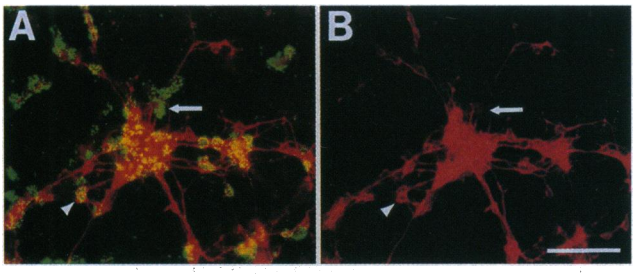

FIG. 4. Continual neurogenesis in neuronal colonies at 7 days *in vitro*. Co-cultures of purified OE progenitors over growth-arrested stroma were pulsed for 10 hr with 1 μCi/ml ^{3}H-TdR at 6 days *in vitro*, then chased with cold thymidine (50 μM) for 24 hr prior to fixation and processing for NCAM immunoreactivity and autoradiography (9). A neuronal colony is shown in a double exposure for NCAM immunoreactivity (red) and silver grains indicating ^{3}H-TdR incorporation. Silver grains appear green if present in an NCAM$^-$ cell, yellow if present in an NCAM$^+$ cell. Some dividing cells have generated post-mitotic, NCAM$^+$ neurons (arrowhead), but a significant number of NCAM$^-$, ^{3}H-TdR$^+$ cells remain (arrow). For clarity, the same colony, exposed for NCAM immunoreactivity alone, is shown in *B*. (Bar = 50 μm.) Control experiments in which ^{3}H-TdR labeling was performed in combination with X-gal staining showed that all ^{3}H-TdR-positive cells in labeled colonies were β-gal-positive, and that the incidence of ^{3}H-TdR-labeled stromal cell nuclei in the feeder layers was extremely low [0.73 (± 0.19) labeled nuclei/250,000 μm^2]. When labeled stromal cell nuclei were observed, their diameters were found to be much larger than those of olfactory neuronal cell bodies (30–40 μm vs. 6–8 μm). These findings make it highly unlikely that ^{3}H-TdR incorporation by stromal cells in the feeder layers of these cultures could account for the observed labeling in neuronal colonies.

a large excess (≈20-fold) of neuronal cells isolated from 14-hr CD-1 OE suspension cultures was added onto the ROSA26 progenitors 1–2 hr after the progenitors were plated. The neuronal cell fraction from such OE suspension cultures is known to consist of ≈75% differentiated ORNs and 25% ORN progenitors (11); thus, about a 15-fold excess of differentiated neurons was actually added to progenitor cultures at the time of plating. In control experiments, a 20-fold excess of dissociated CD-1 OE stroma cells was added to ROSA26 progenitor cultures. Cultures were grown for 6–7 days, fixed, processed for X-gal histochemistry, and scored for each colony type.

As shown in Table 1, addition of neuronal cells decreased the incidence of neuronal colonies by more than 50% [$P <$ 0.02, paired t test (20)]. Addition of stroma cells had no significant effect on frequency of neuronal colony formation. Interestingly, inhibition was specific for the formation of neuronal colonies; formation of other colony types was not affected significantly. In addition, the sizes of neuronal colonies did not appear to decrease in cultures to which excess neurons had been added (not shown). Data from two typical experiments are graphed in Fig. 5 to illustrate relative frequency of neuronal CFUs formed under the two conditions. The frequency of neuronal CFU formation is ≈1 in 3600 of plated purified progenitors under normal progenitor/stroma co-culture conditions, but when an excess of neurons is added this frequency drops 2.5-fold, to ≈1 in 9000.

Table 1. Effect of added cells on development of different colony types

Exp. No.	Control	Neuronal cells added	Stroma cells added	% change	
Neuronal colonies*					
1	23	10		−56.5	
2	12	6		−50.0	
3†	16	6		−62.5	
7†	15	10		−33.3	
Mean				−50.6	$P = 0.019$
4	22		28	+27.3	
5‡	14.4		14.4	0	
6†	18		18	0	
7†	15		20	+33.3	
Mean				+15.2	$P = 0.184$
Spindle-shaped cell colonies*					
1	13	19		+46.2	
2	1	1		0	
3†	10	16		+60.0	
7†	6.7	6.7		0	
Mean				+26.6	$P = 0.182$
4	2		1	−50.0	
5‡	4.8		2.4	−50.0	
6†	<1§		4	>300	
7†	6.7		5	−25.0	
Mean				>+43.8	$P > 0.694$
Medium-round cell colonies*					
1	49	33		−32.7	
2	24	22		−8.3	
3†	24	36		+50.0	
7†	83.3	56.7		−32.0	
Mean				−5.8	$P = 0.403$
4	25		32	+28.0	
5‡	19.2		19.2	0	
6†	32		18	−43.8	
7†	83.3		91.7	+10.0	
Mean				− 1.5	$P = 0.950$
Fibroblastic cell colonies*					
1	45	71		+57.8	
2	12	8		−33.3	
3†	8	8		0	
7†	45	50		+11.1	
Mean				+8.9	$P = 0.386$
4	8		15	+87.5	
5‡	16.8		14.4	−14.3	
6†	20		12	−40.0	
7†	45		16.7	−63.0	
Mean				−7.5	$P = 0.366$

Approximately 2 × 10^4 CD-1 OE neuronal cells or mitomycin-treated (2 hr) stroma cells were added to each well 1.5 hr after plating ≈1000 purified ROSA26 progenitors onto growth-arrested CD-1 stroma feeder layers. Plating efficiency of ROSA26 progenitors, when plated alone or with cells added, was determined in a given experiment by examining two wells in each condition, which were fixed 6–8 hr after plating, processed for X-gal histochemistry, and total X-gal$^+$ cell number counted; the number of ROSA26 progenitors present never varied by >2% in any condition. For analysis of effects of added cells on colony formation cultures were fixed and processed for X-gal staining after 6–7 days and different colonies counted. Addition of neuronal cells inhibited formation of neuronal colonies significantly ($P = 0.19$, paired t test) (20); addition of stroma cells had no significant effect.

*Colony numbers have been normalized to 96,000 input cells, the number normally plated in a 96-well plate for each test condition.

†Forty-eight wells tested in each condition.

‡Forty wells tested in each condition.

§No spindle-shaped cell colonies were observed in the 48 wells tested.

DISCUSSION

As a system for studying cellular and molecular events regulating neurogenesis, mammalian OE has the advantages that it produces only a single type of neuron (the ORN) and retains the capacity for continual neurogenesis throughout life (2, 6, 21). Methods allowing identification and isolation of specific OE cell types, *in vitro* and *in vivo*, have led to a view of the OE neuronal lineage in which at least two distinct stages of neuronal transit amplifying progenitors are interposed between post-mitotic ORNs and the stem cell assumed to be ultimately responsible for continual neurogenesis in this tissue (22). They have also made it possible to screen for mechanisms and specific factors that influence proliferation, differentition, and survival of these different cell types, and mounting evidence indicates that cell number at each identified stage in this lineage can be regulated by extrinsic factors (6, 9, 12, 23, 24). However, identification of the neuronal stem cell in this system, and characterization of factors influencing its development, have so far been problematic.

In this study, we sought to characterize OE neuronal stem cells by purifying neuronal progenitors from embryonic mouse OE and examining their development in defined, serum-free culture. This strategy revealed that the vast majority of progenitors act as neuronal transit amplifying cells in isolated culture, just as they do *in vivo* and in explant cultures of OE; they rapidly give rise to differentiated ORNs, which then die within a few days in the

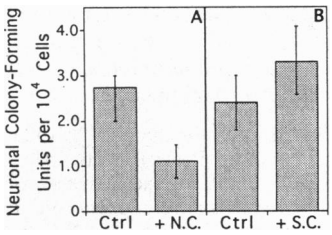

FIG. 5. Effect of added neuronal and stroma cells on neuronal colony formation. Two typical experiments, taken from those shown in Table 1, in which a large excess of neuronal (*A*) or stroma (*B*) cells were added to purified ROSA26 progenitor cultures shortly after plating. The number of wells containing one or more neuronal colonies was counted, and the frequency of neuronal colony formation was calculated from the zero-order term of the Poisson equation (17). Error bars reflect standard deviations resulting from sampling error.

absence of target tissue or appropriate trophic support (Fig. 1) (6, 9, 10, 12). However, co-culturing purified progenitors with stroma cells, isolated from turbinate tissue underlying the OE and with which OE progenitors are normally associated *in vivo*, permitted development of proliferative colonies from a small fraction of cells (CFUs). Colonies arose at a frequency of about 1 in 1000 purified progenitor cells plated (Fig. 3). A morphologically and antigenically identifiable subset of colonies retained the capacity for neurogenesis as late as 7 days *in vitro* (Figs. 2 and 4); these neuronal colonies arose at a frequency of about 1 in 3600 progenitor cells plated, and their development was specifically inhibited by adding an excess of differentiated ORNs to progenitor cell cultures shortly after plating (Table 1, Fig. 5).

Colony Types Arising in Progenitor Cell Cultures. One possible explanation for the observation of four distinct colony types (Fig. 2) is that a multi-potent stem cell of the OE, present at a frequency of about 1 in 1000 purified progenitors, gives rise to every proliferative colony type. We believe that this is unlikely, however. First, the fibroblastic colony type may be a contaminant of progenitor cell cultures. Evidence in support of this idea includes the following: (*i*) The relative proportion of fibroblastic colonies varied widely in different experiments (Table 1). (*ii*) Morphology of cells in fibroblastic colonies was identical to that of stromal fibroblasts. (*iii*) Control experiments in which stromal cells were prepared from ROSA26 turbinates and plated in serial dilutions onto growth-arrested CD-1 feeder layers showed a linear relationship between number of cells plated and formation of fibroblastic colonies, with 1 colony formed for every 10 cells plated (data not shown). Contamination of ROSA26 progenitor cell preparations with stromal fibroblasts at a level of <0.1% of total cells would therefore be sufficient to account for the number of fibroblastic colonies observed in the present study (Table 1). Despite the fact that every precaution was taken to remove stromal fibroblasts from OE during purification, contamination at such a low level cannot be ruled out. Taken together, these observations are consistent with the conclusion that the presence of fibroblastic colonies is due to a low (<0.1%) and variable level of contamination of progenitor cell preparations with stromal fibroblasts.

If fibroblastic colonies are excluded from analysis, the relative proportion of the three other colony types was reasonably consistent among experiments, with colonies of the medium-round type occurring most frequently (59.3 ± 4.2%, mean ± SEM of seven experiments), followed by neuronal colonies (32.03 ± 3.6%). Colonies of spindle-shaped cells were rarest, comprising about 9% of colonies (8.71 ± 2.8%) and occasionally failing to develop at all (Table 1, Exp. 6). These proportions may reflect the relative abundance of three rare progenitor cell types in the panned progenitor preparations, or all three cell types might arise from a single multi-potent cell. However, even at the lowest densities plated, where colonies arose at <1 per well (e.g., Fig. 3, Table 1), we never observed colonies that were a mixture of cell types. If all colony cell types arose from a single progenitor, mixed colonies should be seen at least some of the time. Instead, the observations suggest that different colony morphologies resulted from clonal expansion of different progenitor cell types.

The spindle-shaped cells that comprise the rarest colony types bear a morphological resemblance to cultured olfactory Schwann or ensheathing cells (18, 19). In this regard, it is interesting that previous studies have reported evidence that olfactory Schwann cell progenitors may be present in embryonic OE (19, 25). The results of this study lend indirect support to this idea. In addition, if the spindle-shaped cells are in fact olfactory Schwann cells, these results suggest that their progenitors are the rarest neural progenitor cell type in the OE. The identity of the "medium-round" cells, which comprise the most frequent colony type, is unknown. Daily observations of these colonies using phase-contrast microscopy, in addition to retrospective analysis with X-gal histochemistry and NCAM immunocytochemistry, have never yielded evidence that they give rise to neurons (not shown). These cells may be progenitors of the supporting or sustentacular cells of the OE. Making this identification will require development of a marker specific for embryonic mouse sustentacular cells, similar to rat-specific markers developed by others (26).

Is the Neuronal CFU the Neuronal Stem Cell of the OE? Colonies that contain small, round, neurite-bearing cells when viewed with X-gal histochemistry have been shown to contain both $NCAM^+$ ORNs and proliferating $NCAM^-$ progenitors at 7 days in culture (Figs. 2 and 4). We hypothesize that these neuronal colonies arise from a neuronal CFU (colony-forming cell or unit), analogous to CFUs thought to be indicative of stem cells in retrospective analyses of hematopoietic development (27). Defining characteristics of stem cells include a high capacity for self-renewal and the ability to give rise to a lineage whose endpoint is a terminally-differentiated cell (28–30). The fact that neuronal colonies produce differentiated ORNs as late as 7 days *in vitro*, yet still contain proliferating cells, is consistent with the presence of a progenitor cell with these characteristics. Longer-term experiments will be required, however, to determine the extent of the capacity of neuronal CFUs for self-renewal. It is likely that the present culture conditions will need to be modified before longer-term studies can be carried out, since we find that, in reduced calcium and serum-free culture (see *Materials and Methods*) the survival of stromal cells for longer than 7 days is poor.

An additional characteristic of stem cells in some permanently renewing systems, such as the hematopoietic system, is that they are quite rare (27). In fact, our estimate of 1 in 3600 progenitors for neuronal CFU frequency is on the order of that observed for spleen CFUs isolated from bone marrow (≈1 in 3800) (31), although somewhat lower than estimates of stem-cell frequency in epidermis or fetal liver (32, 33). However, the lengthy procedure required for purifying progenitors could easily cause damage to cells, and it is likely that we have not yet optimized conditions for culturing neuronal CFUs. Thus, in the OE, neuronal CFUs may be more abundant than our current estimate, and the system we describe here should prove useful for identifying factors important in regulating their survival and proliferation. In any case, the rarity of neuronal colonies suggests a system dependent upon proliferation of transit amplifying cells for the bulk of its regenerative capacity, in keeping with what is known concerning the multi-step nature of neurogenesis in the OE (9, 10).

This study indicates that cells derived from stroma subjacent to the OE provide factors necessary for neuronal CFU development. Stromal cells are known to play a crucial role in regulating survival of stem cells in hematopoietic development. Much like OE progenitors, hematopoietic progenitors grown in the absence of added factors survive only a short time in culture. However, bone marrow-derived stromal cells support survival of self-renewing stem cells and development of myeloid, erythroid, and lymphoid lineages in culture (34). Co-culture with stroma is known to be crucial for *in vitro* growth, differentiation, and maturation of epithelia (e.g., refs. 35 and 36), and epidermal stem cells require fibroblast feeder

layers for development *in vitro* (33). Identification of a stroma-derived factor(s) crucial for neuronal progenitor renewal in the OE will provide important information about molecular regulation of neuron production, and may also provide clues as to the identity of the factors that permit life-long neuron renewal in the OE, in contrast to other areas of the nervous system.

Differentiated Neurons Inhibit the Development of Neuronal Colonies. In the OE *in vivo*, progenitor cells proliferate when ORNs overlying them undergo cell death and degenerate, suggesting that ORNs might provide a negative feedback signal that inhibits precursor proliferation (8, 10, 12, 22). Consistent with this hypothesis, we observed that addition of an excess of differentiated ORNs to progenitor cell cultures decreased the formation of neuronal colonies. Indeed, while performing experiments to ascertain the frequency of colony-forming progenitors in progenitor/stroma co-cultures (Fig. 3), we had noted a trend for CFU frequency to decrease as progenitors were plated at increasing densities, although this trend was not statistically significant (data not shown). It seems possible that newly differentiating ORNs, generated within the first 24 hr of culture by >90% of purified progenitors in progenitor/stroma co-cultures (compare with Fig. 1*A*), might themselves partially suppress the formation of neuronal colonies.

When an excess of differentiated ORNs was plated along with purified progenitors, no significant effects on the numbers of non-neuronal colony types were seen (Table 1, Fig. 5), indicating that inhibition of colony formation was specific for neuronal colonies. This suggests that inhibition is not simply the result of added cells depleting the medium of nutrients, a view further supported by the absence of an inhibitory effect when an excess of stromal cells was plated with progenitors (Table 1, Fig. 5). Thus, differentiated ORNs appear to provide a signal that inhibits development of neuronal CFUs. One other example of feedback regulation of neuron production has been reported: In larval *Xenopus* retina, ablation of cells *in vivo* by intraocular injection of 6-hydroxydopamine or the excitotoxin kainic acid results in preferential production of new cells of the appropriate type (37, 38).

There are two possible explanations—not mutually exclusive—for the observation that fewer neuronal colonies are observed when progenitors are cultured in the presence of an excess of differentiated neurons: Differentiated neurons may produce a signal that inhibits proliferation and/or survival of neuronal CFUs; alternatively, the presence of differentiated neurons may cause neuronal CFUs to adopt another fate. There is precedent for neuron-derived factors affecting fate choices of neural progenitors. For example, Shah and colleagues (39) have shown that growth of neural crest cultures in glial growth factor biases these cells toward a glial, as opposed to neuronal, fate and that crest-derived neurons express both glial growth factor protein and mRNA. In our experiments, we noted that addition of excess ORNs to OE progenitor cell cultures resulted in an increase in the number of colonies of spindle-shaped cells (which resemble olfactory Schwann cells morphologically) in two of four experiments; however, this result was not statistically significant (Table 1). It will be interesting to determine if ORNs produce glial growth factor, and whether addition of glial growth factor to ORN progenitor cell cultures results in an increase in the frequency at which spindle-shaped cell colonies develop.

It is noteworthy that, in this study, addition of excess ORNs to OE progenitor cell cultures resulted in a decrease in the number, rather than the size, of neuronal colonies (Table 1, and data not shown). Such a result would be predicted if the signal produced by differentiated neurons acts upon cells at a very early progenitor stage, causing a fraction of them either to die or to stop producing the downstream neuronal transit amplifying cells that generate ORNs. Uncovering the mechanisms that underlie feedback regulation of neuron production in the OE may be important for understanding why neuronal production in most other parts of the nervous system gradually slows during development, and eventually halts (e.g., ref. 40). The results of the present study suggest that such slowing may result, at least in part, from inhibition by differentiated neurons of proliferation and/or survival of cells at the earliest stages in neuronal lineages.

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