Isolation and *In Vitro* Differentiation of Conditionally Immortalized Murine Olfactory Receptor Neurons

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Two major challenges exist in our understanding of the olfactory system. One concerns the enormous combinatorial code underlying odorant discrimination by odorant receptors. The other relates to neurogenesis and neuronal development in the olfactory epithelium. To address these issues, continuous cell cultures containing olfactory receptor neurons (ORNs) were obtained from olfactory epithelia of H-2K^b-tsA58 transgenic mice. ORNs were detected and characterized by immunocytochemistry, RT-PCR, and Western blot for the markers G α_{olf} , adenylyl cyclase III, the olfactory cyclic nucleotide-gated channel subunits, and olfactory marker protein. In culture, epidermal growth factor and nerve growth factor stimulated proliferation, and brain-derived neurotrophic factor and neurotrophin-3 induced cellular maturation.

Clonal cell lines were isolated by fluorescence-activated cell sorting with anti-neural cell adhesion molecule antibodies, and

of 144 single cells plated, 39 clones were expanded, propagated, and stored in liquid nitrogen. All attempts at recovery of clonal lines from frozen stocks have been successful. The most thoroughly characterized clone, 3NA12, expressed ORN markers and responded to stimulation by single odorants. Each odorant activated ~1% of cells in a clonal line, and this suggests that many different odorant receptors may be expressed by these clonal cells. Therefore, these cell lines and the method by which they have been obtained represent a significant advance in the generation of olfactory cell cultures and provide a system to investigate odorant coding and olfactory neurogenesis.

Key words: olfactory receptor neurons; H-2K^b-tsA58 transgenic mouse; cell lines; immortalization; proliferation; trophic factors

Several *in vitro* approaches have been used to address both odorant-coding and neuronal development issues in the olfactory system. These include receptor transfection studies (Raming et al., 1993; Krautwurst et al., 1998), primary cultures of olfactory epithelial slices (Gong et al., 1996), and primary cultures of olfactory receptor neurons (ORNs) dissociated by enzymatic digestion of olfactory epithelia (Calof and Chikaraishi, 1989; Ronnett et al., 1991; Vargas and Lucero, 1999). However, these studies have been hampered by inefficient odorant-receptor-protein translocation to the plasma membrane in heterologous systems (McClintock et al., 1997) or by limited survival of ORNs beyond 7 d in culture (Ronnett et al., 1991; Vargas and Lucero, 1999). To overcome the survival issue, there have been several attempts to develop “immortal” olfactory cell lines.

Immortal olfactory receptor neuron cell lines fall into two categories. In one, immortalization occurred by chance (Wolozin et al., 1992; Vannelli et al., 1995), and in the other, immortalization occurred after planned manipulations (Largent et al., 1993; MacDonald et al., 1996b). In the first category, cell lines were isolated from olfactory epithelia of adult human and aborted human fetuses. These cells expressed olfactory-specific proteins and gave functional responses to odorant stimulation in biochemical and fluorescence-based assays, respectively (Wolozin et al., 1992; Vannelli et al., 1995). In the second category, immortalization was induced by one of two techniques. In the first technique, cells were immortalized by expression of the Simian virus 40 large tumor antigen (TAG). Cells were cloned from a mouse in which the TAG was under the control of the regulatory elements of the olfactory marker protein gene (Largent et al., 1993). These cells expressed a growth-associated neuronal marker and underwent morphological changes in response to “differentiating” agents. In a recent study, a conditionally immortalized rat ORN cell line was isolated using transfected tsA58, a temperature-sensitive mutant of the TAG (Murrell and Hunter, 1999). Functional responses from transfected, exogenous odorant receptors could be observed in these cells, but single-cell cloning attempts were unsuccessful, and endogenous functional responses to odorants could not be demonstrated. In the second technique, the epithelium of bulbectomized adult mice was transfected with the immortalizing oncogene n-myc (MacDonald et al., 1996b). The presence of mRNA encoding olfactory markers was detected in these cells, but for neither these cells nor the cells of Largent et al. (1993) were functional data available.

To develop a method that reliably yields clonal lines of murine

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ORNs, we have generated immortal cell cultures from the H-2K^b-tsA58 transgenic mouse (Jat et al., 1991). The genome of this mouse harbors the γ -interferon-inducible mouse major histocompatibility complex promoter sequence, situated upstream of the temperature-sensitive TAG. *In situ*, this transgene is inactive, but when cells are isolated from this mouse and cultured in the presence of γ -interferon and at 33°C (permissive conditions), the cells exhibit a conditional immortalization. In nonpermissive conditions (i.e., at 37°C in the absence of interferon) the cells differentiate (Chambers et al., 1993; Barber et al., 1997). Therefore, this mouse provides an ideal source for the generation of ORN cell lines. We show here that clonal ORN cell lines can be isolated from this mouse and that they can be maintained and passaged in permissive culture conditions. Odorant-stimulated responses can be observed in these cells, and phenotypic changes consistent with cell differentiation occur in nonpermissive culture conditions. These cells can now be used to study ORN development and, it is hoped, will permit the efficient, functional expression of exogenous odorant receptors.

MATERIALS AND METHODS

Cell culture. To keep the amount of expressed TAG to the minimum required for immortalization (Noble et al., 1995), heterozygous offspring from homozygous male H-2K^b-tsA58 transgenic and C57Bl female mice (Charles River Laboratories, Wilmington MA; and The Jackson Laboratory, Bar Harbor, ME, respectively) were used to generate the conditionally immortal neuronal cell culture. Cells were isolated in a procedure modified from a previously established technique (Ronnelt et al., 1991). Briefly, postnatal day 1–3 animals were decapitated, and the heads were sectioned longitudinally. The olfactory epithelium and nasal septum were removed and placed in an Eppendorf tube containing 500 μ l of culture medium. The culture medium consisted of minimum essential medium in which the L-valine has been replaced by D-valine (MDV). This was supplemented with 10% FBS, 4 mM glutamine, kanamycin (100 μ g/ml), gentamycin (50 U/ml), and amphotericin B (2.5 μ g/ml; all from Life Technologies, Gaithersburg, MD). The MDV medium was chosen because fibroblasts lack D-amino acid oxidase, and the reduction of L-valine inhibits fibroblast survival and growth (Gilbert et al., 1986). Tissue was centrifuged at low speed for 2 min, and then most of the medium was removed. The cell pellet was chopped briefly with a pair of fine-point scissors before being resuspended in supplemented cell culture medium. The resultant cell clumps and cell suspension were plated out onto two- or four-chamber glass slides (Nunc, Naperville, IL) or plastic dishes (Fisher Scientific, Pittsburgh, PA). To enhance ORN adhesion, all cell culture materials were pretreated with laminin (0.125 mg/ml in serum-free media; Collaborative Biomedical Products, Bedford, MA) for at least 12 hr before plating. Cells were maintained in culture at 33°C (the permissive temperature for the SV40 TAG), and, to stimulate transcription of the transgene, the culture medium was supplemented with murine γ -interferon (40 U/ml; Genzyme, Cambridge, MA). Provisionally, in permissive conditions, the cell culture medium was supplemented with epidermal growth factor (EGF, 20 ng/ml; Life Technologies) and 2.5 S nerve growth factor (NGF, 10 ng/ml; Life Technologies) before thorough characterizations of their effects were made in later experiments.

To determine optimum conditions for cell survival, growth, differentiation, or maturation, numerous combinations of culture conditions were tested. These included (1) removal of the olfactory epithelium from newborn, 4-week-old, and sexually mature animals, (2) subjecting tissue to digestion by trypsin (Life Technologies; 0.5 gm/l for 60 min at 37°C), (3) addition of growth factors to the culture medium, and (4) the use of diverse culture media. Cells were plated out and fed in each of the following media: Neurobasal/B27, Neurobasal/B27/serum, MDV/dialyzed serum, and MDV/FBS (all from Life Technologies), all in the presence of interferon (40 U/ml), EGF (20 ng/ml), and NGF (10 ng/ml). Subsequently, the effects of different concentrations of interferon, EGF, NGF, brain-derived neurotrophic factor (BDNF; Peprotech, Rocky Hill, NJ) and neurotrophin-3 (NT-3; Peprotech) were also tested. On the basis of those experiments, interferon (10 U/ml), EGF (10 ng/ml), and NGF (10 ng/ml) were routinely added to the culture medium for all cells maintained in permissive culture conditions (33°C).

Cells were always maintained in permissive culture conditions except

when attempts were made to obtain a differentiated phenotype through transgene inactivation. In this case, cells were incubated in nonpermissive culture conditions, i.e., at 37°C in γ -interferon-free media for 7 d, before the effects of transgene inactivation were determined. The effects of EGF, NGF, BDNF, and NT-3 in promoting cell maturation and differentiation were assessed, and, on the basis of those experiments, BDNF and NT-3 were routinely added to the culture medium for cells in nonpermissive culture conditions. In both culture environments, the air was kept humidified and contained 5% CO₂. Cells from homozygous C57Bl mice were used as controls and did not survive beyond a few days.

Immunocytochemistry. Cells were rinsed in PBS, pH 7.4, at 37°C and fixed in either ice-cold methanol (10 min at –20°C) or fresh paraformaldehyde (2% in PBS for 20 min on ice). After fixation, cells were permeabilized by incubation with 0.1% Triton X-100 in PBS for 20 min on ice. Cells were rinsed (three times for 5 min each in PBS) and blocked with 10% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA) for 60 min. Incubation with primary antibodies was performed in 5% NDS in PBS at 4°C overnight. The following primary antibodies and dilutions were used: anti-neuron-specific tubulin (NST, 1:1000; Babco, Richmond, CA), anti-gial fibrillary acidic protein (GFAP, 1:800; Dako, Carpinteria, CA), anti-neural cell adhesion molecule (NCAM, 1:100; Chemicon, Temecula, CA), anti-G α_{olf} (1:1000), anti-adenylate cyclase type III (ACIII, 1:1000; both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-OE1, a transcription factor in the olfactory epithelium (1:1000; a gift from R. Reed, Johns Hopkins University School of Medicine, Department of Molecular Biology and Genetics), anti-olfactory marker protein (OMP) (1:1000; a gift from F. Margolis, University of Maryland Medical School, Baltimore, MD), anti-neuron-specific enolase (NSE, 1:2; Incstar Corp., Stillwater, MN), and anti-p75 NGF receptor (p75 NGFR, 1:1000; Boehringer Mannheim, Indianapolis, IN). The following day, cells were rinsed (three times for 5 min each) with PBS and incubated sequentially with the appropriate fluorescein- or rhodamine-coupled secondary antibodies (1:50 and 1:100, respectively) in 5% NDS in PBS for 60 min. Subsequently, cells were rinsed and mounted in Aquamount (VWR, West Chester, PA) and photographed using 100 ASA color Ektachrome film (Eastman Kodak, Rochester, NY).

PCR analysis. PCR was used to confirm the presence of olfactory-specific markers in the cells obtained from the H-2K^b-tsA58 transgenic mouse. Using the MicroFast Track kit (Invitrogen, Carlsbad, CA), mRNA was isolated from cell cultures in both permissive and nonpermissive conditions and from olfactory tissue (postnatal day 2). cDNA was produced from the mRNA using Superscript Reverse Transcriptase II (Life Technologies) with a mixture of random hexamer and oligo-dT primers. Enzyme-free reactions were performed as controls for the following PCR analysis. The resulting cDNA was amplified by PCR using the Expand high-fidelity PCR system (Boehringer Mannheim) with primers for the following markers: tubulin, 45°C, 5'-TGCTCATC-AGCAAGATCCGAG, 3'-GGAATGGCACCATTGTCACAG; OE1, 54°C, 5'-GAAGCCAACAGCGAAAAGAC, 3'-CTTGT-TTTGTCAT-GGAGTCG; OCNC1, 56°C, 5'-CTATTTTGTGGTATGG-CTGGTGC, 3'-CAAGCATCCAGTGGATGATGAC; OCNC2, 65°C, 5'-GTGCT-AAAGCTCCAGCCCCAGAC, 3'-AGCCAGACTCTGTGGCTCTCT-G α_{olf} , 50°C, 5'-AGAGATGAGAGAAAATGG, 3'-TGCTCTT-GTAACCTTGGATC; ACIII, 56°C, 5'-TGGCAGCACC-TGGCTGAC, 3'-GGGGCAGTGTAACAGAGGA; and OMP, 60°C, 5'-AAGGTCACC-ATCACGGGCAC, 3'-TTAGGTTGGCATTCTCCAC.

The amplification protocol was 94°C for 5 min (94°C for 1 min, 8°C for 1 min, and 72°C for 1 min) for 35 cycles and 72°C for 10 min final extension, where δ is the primer-specific annealing temperature listed above. Products were ligated into the pCR2.1 vector supplied with the TA cloning kit (Invitrogen) and transformed. Colonies were screened by PCR, and positive clones were grown up as minipreps for plasmid isolation (Qiagen, Valencia, CA). The cDNAs obtained were sequenced in the Howard Hughes Medical Institute Sequencing Laboratory to confirm the presence of the correct insert. All protocols were performed according to the manufacturers' instructions.

Western blot analysis. Cells were scraped into a cold isolation buffer containing 20 mM Na-(N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 10 mM mannitol, and 1% Triton X-100, pH 7.4. The isolation buffer was supplemented with phenylmethylsulfonyl fluoride (30 μ g/ml), leupeptin (2 μ g/ml), benzamide (16 μ g/ml), pepstatin (2 μ g/ml), and lima bean trypsin inhibitor (50 μ g/ml) to prevent protease activity. After five freeze-thaw cycles, cell extracts were centrifuged at 2000 $\times$ g for 5 min to pellet nuclei and cell debris. Supernatants were assayed for protein concentration using the bicinchoninic acid protein reagent kit (Pierce, Rockford, IL).

Samples from cell extracts were prepared for electrophoresis by boiling for 5 min in 125 mM Tris loading buffer, pH 6.8, containing 1% SDS and 3.2% β -mercaptoethanol. Proteins (15 μ g/lane) were separated on SDS-polyacrylamide gels containing 6% acrylamide (0.19% *N,N'*-methylene-bisacrylamide), 10% acrylamide (0.27% *N,N'*-methylene-bisacrylamide), or 16.5% acrylamide (0.67% *N,N'*-methylene-bisacrylamide) alongside molecular weight standards (Amersham, Arlington Heights, IL). Proteins were then transferred to Immobilon-P membranes (Millipore, Bedford, MA) in 25 mM Tris, 200 mM glycine, pH 8.5, and 20% methanol for 1.5 hr at 500 mA. Blots were blocked with 5% nonfat dry milk diluted in 50 mM Tris-HCl and 150 mM NaCl, pH 7.5, containing 0.05% Tween 20 (TTBS) for up to 45 min. Subsequently, blots were incubated in TTBS with the appropriate dilution of primary antibody for 2 hr at room temperature. To probe the transferred proteins, the following antisera were used at the indicated dilutions: polyclonal rabbit antisera against OCNC1 (Bradley et al., 1997; 1:1000), ACIII and $G\alpha_{olf}$ (1:500 and 1:1000, respectively), polyclonal goat antisera against OMP (1:2500), and monoclonal mouse ascites against the large T antigen (a gift from T. Kelly, Johns Hopkins University School of Medicine, Department of Molecular Biology and Genetics; 1:500). After washing, blots were incubated for 45 min with HRP-conjugated donkey anti-rabbit IgG (1:10,000; Amersham), HRP-conjugated rabbit anti-mouse IgG (1:5000; Amersham), or HRP-conjugated donkey anti-goat IgG antibodies (1:10,000; Jackson ImmunoResearch) and visualized using the Enhanced Chemiluminescence kit (Amersham). Exposure times ranged from 1 to 10 min. Blots were then stripped by incubation for 20 min at 50°C in 62.5 mM Tris HCl, pH 6.7, 2% SDS, and 100 mM β -mercaptoethanol in a shaking water bath and reprobed with monoclonal mouse hybridoma media against monomeric actin (JLA20, Developmental Studies Hybridoma Bank; 1:500) to control for protein loading. Visualization was achieved as described above.

Fluorescence-activated cell sorting. Passage 3 cells from one 100 mm plate were subjected to a brief enzymatic (trypsin) digestion before antibody labeling and fluorescence-activated cell sorting (FACS). Cells were incubated sequentially with a rabbit anti-NCAM antibody and with an FITC-coupled donkey anti-rabbit secondary antibody for 45 min each. Negative control and background experiments (two 60 mm dishes) were performed by omitting either both labeling steps or the primary labeling step, respectively. All incubations were performed on ice in the presence of 10% normal donkey serum. Cell sorting was performed on a FAC-StarPLUS, modified with Turbosort (Becton Dickinson, Bedford, MA), and gating was based on fluorescence intensity, subject to cell size (forward scatter) and granularity (side scatter) restrictions. Single cells were transferred into laminin-coated 96-well plates and maintained in culture as described previously. Subsequently, cells were passaged into single wells of a 12-well plate and then into a 10-cm dish before storage in liquid nitrogen. Clones have been named on the basis of the well from which they were obtained. For example, clone 3NA12 was obtained from well A12 in the third (3) plate, which contained cells sorted on the basis of NCAM immunoreactivity.

Calcium imaging. Cloned olfactory receptor neurons were plated onto glass coverslips up to 1 week before imaging. On the day of analysis, the culture medium was removed and replaced with MDV containing 2 μ M fura-2 AM (Molecular Probes, Eugene, OR) and 0.2% Pluronic F-127 (Sigma, St. Louis, MO) dissolved in DMSO. Cells were incubated at appropriate culture temperatures (33 or 37°C) for at least 30 min, rinsed, and allowed to equilibrate in medium for 30 min before experiments. Additionally, to ensure that a response could not be induced mechanically, each experiment was started by washing the cells with bath solution in the same manner used in odorant application. For solution exchange, 5 ml of bath solution or odorant test solution was pipetted manually into the bath (volume, 300 μ l) fitted with a continual suction pump. This ensured complete replacement of the bath solution with the incoming solution without altering bath volume. Single odorants were applied to the cloned cell line 3NA12, and mixtures of odorants were applied to primary cultures of mouse olfactory bulb neurons and rat ORNs. The mixtures consisted of citronellal, pinene, geraniol, and *N*-amyl acetate (mixture 1); acetophenone, heptaldehyde, isovaleric acid, and L-carvone (mixture 2); and ethyl vanillin, helional, isoamyl acetate, and cineole (mixture 3). When single odorants were used, they were taken from mixture 2. Stock solutions of odorants (20 mM in DMSO) were made up every second day and diluted 1:2000 in bath solution (final concentration, 10 μ M each) within seconds of application. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. Calcium imaging was performed as described previously (Grynkiewicz et

al., 1985; Krautwurst et al., 1998), in which ratiometric measurements were obtained using the Zeiss (Thornwood, NY) Attofluor-Ratiovision imaging system on a Zeiss Axiovert 135 microscope fitted with an F Fluor 20 $\times$ /1.30 lens. Cells were illuminated at 340 and 380 nm, and the emission at 510 nm was monitored using an intensified CCD camera. The Attofluor-Ratiovision software was used to derive the Ca^{2+} -dependent ratio. Solutions of $CaCl_2$ (1 mM) and EDTA (1 mM) containing fura-2 pentasodium salt (10 mM; Molecular Probes) were used to provide a two-point calibration of the experimental setup as directed by the instrument manufacturer (Atto Instruments, Rockville, MD).

RESULTS

Cell cultures were obtained from all ages of heterozygous H-2K^b-tsA58 transgenic mice. The proliferation and survival rate was greatest when cells were isolated from early postnatal animals by mechanical dissociation of the epithelia. The optimum basal culture medium was established as MDV and supplemented with nondialyzed FBS. Provisionally, EGF (20 ng/ml) and 2.5 S NGF (10 ng/ml) were added to the culture medium to enhance proliferation and survival. Further quantitative characterization of the effects of NGF and EGF was undertaken later (see below) after the presence of neurons in the cell cultures had been confirmed. Laminin was found to be the most permissive substrate.

Mutually exclusive labeling of cells by NST and GFAP antibodies was observed in both permissive (33°C, with interferon) and nonpermissive (37°C, without interferon) culture conditions in double-labeling experiments (Fig. 1*A–D*). In all experiments, variable numbers of cells that were not stained with either antibody could be observed. These cells, which appeared to vary with culture conditions, passage number, cell density, the antibody used and, potentially, a myriad of other factors, were not investigated further. The presence of neurons in both permissive and nonpermissive culture conditions was confirmed by NCAM labeling. Co-localization, although not absolute overlap, of NST (Fig. 1*E,G*) and NCAM (Fig. 1*F,H*) immunoreactivity was observed through multiple passages and provided further evidence for the existence of a neuronal population. The GFAP-positive (GFAP+) population of cells decreased through multiple passages, with no cells stained with anti-GFAP antibodies by passage 5 in either culture condition (data not shown). This may be attributable to the inability of glia to adhere efficiently to laminin-coated culture dishes (Ronnett et al., 1991).

Optimization of the cell culture conditions

To determine the optimal conditions for cell survival, growth, differentiation, and maturation, cells were cultured in permissive and nonpermissive conditions in the presence of interferon, EGF, NGF, BDNF, and NT-3. These data are summarized in Table 1 and are shown in Figure 2. In permissive conditions, interferon caused a significant increase in the number of NST+ cells, with an EC₅₀ of 0.4 U/ml (Fig. 2*A*). Likewise, in permissive conditions, EGF and NGF increased the number of NST+ cells (EC₅₀ values, 2.0 $\pm$ 1.4 and 2.9 $\pm$ 1.9 ng/ml, respectively; see Fig. 2*B*), but BDNF and NT-3 had no effect (Table 1). The effects of EGF, NGF, BDNF, and NT-3 on cell maturation in nonpermissive culture conditions were assayed by Western blot detection of OMP, a marker of mature olfactory receptor neurons (Margolis, 1988). OMP was most readily detected in cells incubated with NT-3 and, to a lesser degree, was detected in cells incubated with BDNF or NGF (Table 1, Fig. 2*C*). Much weaker OMP immunoreactivity could also be detected in cells grown in the presence of EGF but not in cells maintained in the absence of any growth factors (Fig. 2*C*).

Figure 1. Detection of NST, GFAP, and NCAM in cells from permissive and nonpermissive culture conditions. *A–D*, Cells from the heterogeneous cultures were labeled with primary antibodies for NST and GFAP, and fluorophore-coupled secondary antibodies were used to visualize staining, rhodamine for NST and FITC for GFAP. Mutually exclusive labeling for NST and GFAP was observed in cells grown both in permissive (*A, B*) and nonpermissive (*C, D*) culture conditions. *E–H*, Cells were labeled as above with anti-NST and anti-NCAM antibodies. Co-localization of NST and NCAM labeling is observed in cells grown in both permissive (*E, F*) and nonpermissive (*G, H*) culture conditions. Scale bar, 10 μ m.

On the basis of these data, in all subsequent experiments interferon (10 U/ml), EGF (10 ng/ml), and NGF (10 ng/ml) were added to the culture medium in permissive conditions to enhance proliferation and survival, and BDNF (20 ng/ml) and NT-3 (10 ng/ml) were added to the medium in nonpermissive culture conditions to promote maturation.

Olfactory receptor neurons are detected by immunocytochemistry

To demonstrate whether the neurons detected in this culture system were olfactory in nature, the presence of three olfactory-specific markers was investigated (Fig. 3). Immunoreactivity for $G\alpha_{olf}$ and ACIII, two components of the olfactory second messenger cascade (Jones and Reed, 1989; Bakalyar and Reed, 1990), could be observed in both permissive and nonpermissive conditions (Fig. 3, *A, C* for permissive conditions, *E, G* for nonpermissive conditions, with corresponding co-localization of NST expression shown in *B, D, F, H*). OMP was not detected in permissive culture conditions (data not shown) but was detected in the BDNF- and NT-3-supplemented, nonpermissive conditions (Fig. 3*I*, with *J* showing co-localization of NST). These data

indicate that olfactory receptor neurons are present in our cultures. In addition to the double-labeled cells, some cells in nonpermissive conditions stained only OMP+, $G\alpha_{olf}$ +, and ACIII+ and did not label with NST. This observation is consistent with the staining of NST in the olfactory epithelium, which has been shown to wane in very mature neurons (Roskams et al., 1998).

Olfactory-specific mRNA is detected by RT-PCR

mRNA was isolated from the olfactory epithelia of early postnatal heterozygous mice and from cells cultured in permissive or nonpermissive conditions. After reverse transcription of the mRNA, specific primers for β -tubulin (as a positive control), the olfactory cyclic nucleotide-gated channel subunits OCNC1 and OCNC2, $G\alpha_{olf}$, ACIII, OE1 (an olfactory transcription factor), and OMP were used to amplify the cDNA by PCR. The products had the expected sizes (Fig. 4), and their identities were confirmed by sequencing. To determine the origin of the PCR products, the primers for the two OCNC subunits and OE1 were designed across introns. The sizes of the products from genomic DNA contaminants would have been 0.6, 1.2, and 0.8 kb for OCNC1, OCNC2, and OE1, respectively, rather than the 0.36, 0.38, and 0.1 kb products obtained. mRNA for each of the olfactory markers tested, with the exception of OMP, was present in cells cultured in permissive conditions. In cells maintained in nonpermissive culture conditions, mRNA for each of the markers, including OMP, was detected. These data are consistent with the presence of olfactory receptor neurons in the cultures and a process of cellular maturation and differentiation when cells are switched from permissive to nonpermissive culture conditions.

T antigen and olfactory-specific proteins are detected by Western blot

Further confirmation of the data obtained by immunocytochemistry and PCR analysis was obtained from Western blots. Whole-cell extracts were probed for TAG and for several olfactory-specific markers (Fig. 5). The TAG ran at ~ 70 kDa and was only expressed in cells cultured in permissive conditions (Fig. 5*A*), consistent with the theoretical expectation of TAG expression in cells isolated from the H-2K^b-tsA58 transgenic mouse. The presence of the olfactory proteins OCNC1, ACIII, $G\alpha_{olf}$, and OMP was demonstrated by the bands at the expected sizes of ~ 76 , 170, 44, and 19 kDa, respectively. The band intensities of the olfactory proteins for cells maintained in nonpermissive culture conditions were at least equivalent, if not higher, than those in permissive conditions, even though sample loading was equivalent (as judged by the intensity of actin labeling within each sample pair). Most notable was OMP, which was apparently only expressed in cells grown in nonpermissive conditions (even when the blots were overexposed), confirming the immunocytochemical and PCR results. The presence of OE1 was also investigated, and several bands at different molecular weights were observed (data not shown). These may reflect the presence of the different OE transcription factor isoforms (Wang et al., 1997). These data, together with the immunocytochemistry and the RT-PCR results, indicate that differentiation or maturation of the cells may occur with the change in culture conditions and inactivation of the TAG.

Derivation of clonal cell lines by FACS

It was shown earlier (Fig. 1*G–J*) that NCAM was expressed in the heterogeneous culture system and that it co-localized with NST. Cells were labeled with the anti-NCAM antibody, and attempts were made to isolate independent clones by FACS (data not shown). After labeling, a marked shift of the fluorescence scatter

Table 1. Effects of different growth factors in permissive and nonpermissive culture conditions

	33°C		37°C	
	Normalized no. of NST+ cells	EC ₅₀	Normalized no. of NST+ cells	OMP levels
Control	1.0 ± 0.2		1.0 ± 0.1	–
Interferon (40 U/ml)	2.0 ± 0.2*	0.4 U/ml	1.9 ± 0.1*	ND
EGF (10 ng/ml)	1.7 ± 0.2*	2.0 ng/ml	ND	+/-
NGF (100 ng/ml)	1.5 ± 0.2*	2.9 ng/ml	ND	+++
BDNF (100 ng/ml)	1.3 ± 0.2	NC	1.3 ± 0.2	+++
NT-3 (100 ng/ml)	0.8 ± 0.1	NC	1.4 ± 0.1*	++++++

The effects of different growth factors were assessed by counting cells labeled with the anti-NST antibody. For experiments with interferon, cells were grown in the presence of increasing interferon concentrations and were normalized to the number of NST+ cells cultured in the absence of interferon. For EGF, NGF, BDNF, and NT-3, all experiments were conducted in the presence of 40 U/ml interferon, and the data were normalized to the number of NST+ cells at that point. EC₅₀ values were calculated from sigmoidal curves fitted to the data. NC, The EC₅₀ was not calculated, because no significant effect was observed. OMP levels were estimated from the Western blot, shown in Figure 2C. –, No expression could be observed; ND, no data; number of + symbols corresponds approximately to the level of OMP detected.

**p* < 0.05; determination of statistical significance was made using one-way ANOVA tests.

distribution was observed when compared with controls. One hundred forty-four NCAM+ cells were plated singly into 96-well plates and were cultured in permissive conditions. Thirty-nine proliferative colonies were isolated, expanded, and stored in liquid nitrogen. All attempts at recovery of frozen clonal lines have been successful. One of these clonal lines, 3NA12, has been characterized further. NST, NCAM, NSE, and the p75 NGFR, as well as the olfactory markers Gα_{olf}, ACIII, and OMP, were detected in 3NA12 cells (Fig. 6). Neuronal and olfactory marker co-localization was confirmed in double-labeling experiments (data not shown). In contrast to the heterogeneous cultures in

which nonneuronal cells were observed, all 3NA12 cells expressed markers consistent with the ORN phenotype.

Responses to odorants in a clonal line are observed by calcium imaging

To determine whether cells from the 3NA12 clone were capable of functional responses to odorants, single odorants were applied to cells loaded with fura-2. Preliminary experiments indicated that responses could be observed in cells from both permissive and nonpermissive culture conditions (data not shown). Because the onset of odorant receptor expression is at approximately

Figure 2. Effects of interferon, EGF, NGF, BDNF, and NT-3 on olfactory cell cultures. *A*, Interferon causes a significant, dose-dependent increase in the number of NST+ cells in the heterogeneous culture, with an EC₅₀ of 0.4 U/ml. Cells were incubated at 33°C and were stained with anti-NST and rhodamine-coupled secondary antibodies 7 d after plating. Cell counts were obtained from 10 randomly selected areas in each experiment. Data were normalized to the mean number of NST+ cells in the absence of interferon, and each point represents the mean ± SEM from three experiments. *B*, EGF (●) and NGF (■) both have mitogenic effects in permissive conditions over and above the effect of interferon. NST+ cells were counted as described in *A* and were normalized to baseline levels in the presence of interferon (40 U/ml). EC₅₀ values of 2.9 and 2.0 ng/ml were calculated for effects of EGF and NGF, respectively. Except for the presence of interferon (40 U/ml), all experimental conditions were as in *A*. *C*, Effects of EGF, NGF, BDNF, and NT-3 on OMP expression as determined by Western blot. Cells were maintained in nonpermissive conditions at 37°C in the absence of growth factors (*C*) or in the presence of EGF (*E*), NGF (*N*), BDNF (*B*), or NT-3 (*3*). Samples were isolated from the heterogeneous cell cultures and from olfactory tissue (*T*) and were separated on an SDS-polyacrylamide gel together with a protein ladder (*M*). OMP is a 19 kDa protein and, *in situ*, is expressed only in mature olfactory receptor neurons. OMP was not detected in control cells (*C*) cultured in the absence of growth factors and was only barely detectable in cells incubated in the presence of EGF. Significant expression of OMP was detected from cells grown in the presence of NGF, BDNF, or NT-3, and expression was greatest in cells incubated with NT-3. In all cases, OMP expression in the cultured cells was less than the expression in tissue.

Figure 3. Co-localization of olfactory-specific proteins with NST. Cells from the heterogeneous cultures were labeled with primary antibodies for NST, ACIII, $G\alpha_{olf}$, and OMP. Fluorescent secondary antibodies were used to visualize staining, rhodamine for NST and FITC for $G\alpha_{olf}$, ACIII, and OMP. Co-localization of $G\alpha_{olf}$ and NST labeling was observed in both permissive (*A, B*) and nonpermissive (*C, D*) culture conditions. Similarly, co-localization of ACIII and NST labeling was observed in cells grown in permissive (*E, F*) and nonpermissive (*G, H*) conditions. OMP immunoreactivity (*I*) was only seen in cells maintained in nonpermissive culture conditions, in which co-localization was observed with NST labeling (*J*). Scale bars, 10 μ m (each applies to each horizontal pair of images).

embryonic day 13 in the rat (Strotmann et al., 1995), and each of the components of the signal transduction pathway is present in cells cultured in permissive conditions, cells at permissive conditions were chosen for all subsequent experiments. Four odorants (acetophenone, heptaldehyde, isovaleric acid, and L-carvone) were applied sequentially to a total of 1256 cells in 19 experiments. In 2 experiments (139 cells) no cells responded. In the other 17 experiments, odorant application stimulated an increase in intracellular calcium concentration in 54 cells, the equivalent of 4.3% of cells responding (for examples, see Fig. 7*A–D*). Of the responsive cells, six cells responded to two odorants, but no cells

responded to more than two odorants. An even distribution of responses to each of the different odorants was observed: 15 cells responded to acetophenone, 17 to heptaldehyde, 12 to isovaleric acid, and 16 to L-carvone, approximately equal to 1% of cells responding to each odorant. Repeated applications of the odorants stimulated the cells to respond again, generally with a decrease in the signal observed (for examples, see Fig. 7*E, F*). Control experiments were performed with primary cultures of mouse olfactory bulb neurons and rat ORNs. Both groups of cells were exposed to three odorant mixtures, each containing four odorants. No changes in intracellular calcium concentration were observed after odorant application in 780 olfactory bulb neurons in 12 experiments. In the primary cultured rat ORNs, responses to the odorant mixtures were seen in 12 of 387 cells in six experiments. The frequency of responses in the rat ORN cultures was equivalent to 0.25% of cells responding per odorant, a factor approximately fourfold less than that in 3NA12 cells.

DISCUSSION

Immortalization of olfactory receptor neurons in culture

In the experiments described here, we have defined a reliable method for the production of conditionally immortal ORNs from the H-2K^b-tsA58 transgenic mouse. This method has enabled us to generate many clonal ORN cell lines that express olfactory markers and demonstrate a functional response to odorants. Cells are proliferative when the immortalizing SV40 TAG is expressed, and protein expression patterns consistent with mature ORNs can be induced when the SV40 TAG is degraded or absent. Immortalization occurs as a result of TAG binding to the retinoblastic protein (Rb) and p53. Usually, in nonproliferating cells, growth factors are bound by Rb, and p53 acts as a brake on DNA transcription. However, when these two proteins are bound by TAG, their functions are disrupted. Consequently, transcription can proceed and cell immortalization can occur, as observed in our permissive culture conditions. In nonpermissive conditions (37°C), the TAG is degraded, and cell differentiation begins to occur. In addition to the TAG-mediated immortalization, the survival of our olfactory receptor neuron cultures may in part stem from the fact that neurogenesis naturally occurs postnatally in the olfactory epithelium. Consistent with this idea, hippocampal granule cells, which have been shown to divide *in situ* in marmoset monkeys (Gould et al., 1998), can be conditionally immortalized from fetal H-2K^b-tsA58 mice (Kershaw et al., 1994). Likewise, the non-neuronal cells that have been immortalized from the H-2K^b-tsA58 mouse are proliferative *in situ* (Whitehead et al., 1993).

Characterization of the olfactory receptor neurons and a model for development of the olfactory epithelium

Cells in the heterogeneous cultures of the H-2K^b-tsA58 mouse olfactory epithelium express both neuronal and olfactory markers. All of the olfactory markers that we tested, except OMP, were present in cells cultured in both permissive and nonpermissive conditions. OMP was found only in cells maintained in nonpermissive culture conditions, which may be indicative of induction of a more differentiated phenotype (Margolis, 1988). This is illustrated in Figure 8, in which a schematic diagram depicting a model for ORN development is shown together with the approximate time courses of expression of various proteins. In this model, precursor cells (thought to be globose basal cells; Caggiano et al., 1994), give rise to daughter cells that develop

Figure 4. Amplification of olfactory-specific cDNAs with specific oligonucleotide primers. mRNA from cells grown in permissive (33°) and nonpermissive (37°) culture conditions and from tissue (T) was reverse transcribed (+) or incubated in the absence of enzyme (–). Specific oligonucleotide primers were then used to amplify the cDNA by PCR, and the products were run on 2% agarose gels beside a molecular marker (M). The primer pairs used were specific for β -tubulin, the olfactory cyclic nucleotide-gated channel subunits 1 and 2 (*OCNC1*, *OCNC2*), the type III adenylate cyclase (*ACIII*), the olfactory-specific G-protein α subunit (*Golf*), a transcription factor in the olfactory epithelium (*OE1*), and olfactory marker protein (*OMP*). For each of the primer pairs tested, no product was obtained from reverse transcriptase-free controls. For all other conditions, with the exception *OMP* amplification from cells in permissive conditions, specific products were obtained and confirmed by sequencing.

Figure 5. Detection of large T antigen and olfactory-specific proteins by Western blot. Samples were isolated from the heterogeneous cell cultures grown in permissive (33°) and nonpermissive (37°) conditions and were separated on SDS-polyacrylamide gels (15 μ g protein/lane). Antibodies were used against the large T antigen (TAg), the olfactory cyclic nucleotide-gated channel subunit 1 (*OCNC1*), olfactory marker protein (*OMP*), type III adenylate cyclase (*ACIII*), and the olfactory-specific G-protein α subunit (*G α_{OLF}*). TAg was only detected in cells cultured in permissive conditions, and *OMP* was only detected in cells grown in nonpermissive conditions. *OCNC1*, *ACIII*, and *G α_{OLF}* were each detected in both cell extracts. Equal loading of the protein samples was confirmed by stripping and reprobing blots for monomeric actin.

into mature olfactory receptor neurons. As indicated in Figure 8, the development of mature olfactory receptor neurons can be described immunochemically by the expression of different proteins. For example, NST expression occurs early in development but begins to wane in the most mature olfactory receptor neurons (Roskams et al., 1998). Conversely, *OMP* expression is restricted to mature olfactory receptor neurons (Margolis, 1988), and *G α_{OLF}* and *ACIII* appear to be expressed in both immature and mature neurons. Phenomena broadly similar to these have been observed in our cultures. In permissive culture conditions, all cells that express *G α_{OLF}* or *ACIII* also express NST, but not vice versa. By contrast, in nonpermissive culture conditions, not all cells that express *G α_{OLF}* , *ACIII*, or *OMP* express NST. Therefore, inactiva-

tion of the TAg may be associated with the induction of olfactory receptor neuron maturation in the heterogeneous culture. On the basis of these patterns of labeling, probable developmental windows encompassing our cultures are shown in Figure 8.

Optimization of culture conditions

The data presented here have shown that NGF and EGF enhance the proliferation or survival of the cultured cells in permissive culture conditions, and that BDNF and NT-3 act as differentiating factors. *In situ*, NGF is produced in the olfactory bulb and is transported to the olfactory epithelium (Miwa et al., 1998). NGF receptors have been detected immunohistochemically in the rat and human olfactory epithelium (Balboni et al., 1991; Aiba et al.,

Figure 6. Clone 3NA12 expresses markers of olfactory receptor neuron. Cells from the clone 3NA12 were labeled with primary antibodies for NST, NCAM, NGFR (p75), OMP, NSE, ACIII, and $G\alpha_{olf}$. Labeling was visualized with fluorescent secondary antibodies. *A, B*, NST and NCAM staining, respectively, in cells cultured in permissive conditions. Images are of the same cells, and all cells except one are labeled with both antibodies. This cell (arrow) is only NCAM+ and did not stain NST+; *C*, NST staining as described above for cells cultured in nonpermissive conditions; *D*, p75 NGF receptor staining for cells cultured in nonpermissive conditions; *E*, OMP staining for cells cultured in nonpermissive conditions; *F*, NSE staining for cells cultured in nonpermissive conditions; *G*, ACIII staining for cells cultured in nonpermissive conditions; *H*, $G\alpha_{olf}$ staining for cells cultured in nonpermissive conditions. Scale bars, 10 μ m.

1993) and are upregulated after olfactory nerve transection (Miwa et al., 1993). It has also been shown in tissue culture studies that NGF increases ORN survival and neurite extension (Ronnelt et al., 1991) but has no effect on cell division as measured by radiolabeled DNA precursor uptake (Farbman and Buchholz, 1996). In the present study, NGF enhanced proliferation and survival of our cultures, and it was shown that the p75 NGF receptor was expressed in the clonal 3NA12 cells. These data support the hypothesis that NGF and its associated receptors promote survival of the ORN (Aiba et al., 1993).

EGF and the EGF family member transforming growth factor α are both potent mitogens in the olfactory epithelium, acting on the basal cells that give rise to the ORNs (Mahanthappa and

Figure 7. Functional responses to odorants in the ORN clone 3NA12. Before experiments (2–4 d), cells were plated onto glass coverslips and incubated in permissive conditions. To prepare for stimulation, cells were loaded with fura-2 (2 μ M) for 30 min at 33°C, washed, and allowed to equilibrate for a further 30 min at room temperature. At the beginning of the experiment, cells were washed (*W*) to determine whether the mechanical disturbance associated with changing solution affected intracellular calcium concentrations. Odorants [isovaleric acid (*I*), heptaldehyde (*H*), acetophenone (*A*), and L-carvone (*C*), all 10 μ M] were applied sequentially as a 5 ml bolus into the bath at time points indicated by the arrowheads. Responses to the four odorants can be seen in *A–D*, in which each trace represents data obtained from a single cell. Responses to two odorants by the same cell can be observed in *B* and *D*. Similar responses were observed in other experiments independent of the order of odorant application and in cells maintained in nonpermissive culture conditions. Responses to repeated odorant applications were observed, and examples are shown in panels *E* and *F*.

Schwartz, 1993; Farbman and Buchholz, 1996). EGF receptor mRNA and protein have been detected in the olfactory epithelium and have been localized to the basal cell layer (Balboni et al., 1991; Krishna et al., 1996). Of each of the factors we tested, EGF stimulated the greatest increase of NST+ cells in our heterogeneous cultures in permissive conditions. These are data that support a neurogenic role for the EGF family in the olfactory epithelium (see Fig. 8).

Consistent with mitogenic and survival roles for EGF and NGF, these factors did not have a dramatic effect on cellular maturation. Potential candidates for maturation or differentiating factors include BDNF and NT-3. Expression of BDNF has been detected in the granule cell layer of the olfactory bulb (Guthrie and Gall, 1991) and in the basal cell layer of the olfactory epithelium (Buckland and Cunningham, 1998). BDNF has been shown to promote survival in mouse olfactory epithelium (Holcomb et al., 1995), and the TrkB receptor is expressed by ORNs (Deckner et al., 1993; Holcomb et al., 1995). The TrkC receptor is selectively expressed in mature ORNs, and mRNA encoding NT-3 has been stated to be present in the olfactory epithelium

Figure 8. Schematic representation of the development of the olfactory receptor neuron. The horizontal cells (HC) and the globose basal cells (GBC) are thought to be the precursors of cells in the olfactory epithelium. A mitotic cell (MC), possibly a GBC or its progeny, divides, resulting in the generation of a neuroblast (Nb). The neuroblast then develops into an immature receptor neuron (IRN) and eventually into a mature olfactory receptor neuron (ORN) accompanied by migration of the cell body upward through the olfactory epithelium. Indicated below the diagram is a correlated time course of antigen expression. *Solid lines* indicate confirmed tissue expression, and *dashed lines* indicate an unclear onset of expression. From our experiments, it is suggested that cells in permissive and nonpermissive conditions may be equivalent to cells in the olfactory epithelium as indicated. The points at which EGF, NGF, BDNF, and NT-3 may act are also depicted.

(Roskams et al., 1996). In the present study, BDNF and NT-3 were shown to enhance cellular progression into a “mature, differentiated” phenotype in nonpermissive culture conditions (Fig. 2C), as determined by OMP expression. In the olfactory epithelium, neuronal precursors are TrkB-positive, become TrkB-positive after mitosis, and eventually become TrkC-positive as mature neurons (Roskams et al., 1996). The topographical distribution of these receptors is consistent with a role for the TrkB receptor in stimulating both survival and differentiation of ORNs and for the TrkC receptor in stimulating or maintaining maturation. This hypothesis is supported by the data described here, in which both BDNF and NT-3 enhance the differentiation and maturation state of the cells (see Fig. 8). However, because only four factors have been studied in these experiments, the data are almost certainly incomplete if the whole epithelium *in situ* is to be considered. The fibroblast growth factor family has been proposed to have mitogenic, proliferative, and phenotypic effects on olfactory receptor neurons (DeHamer et al., 1994; MacDonald et al., 1996a; Goldstein et al., 1997), as have ciliary neurotrophic factor, leukemia-inhibitory factor, interleukin-6, and retinoic acid (Farbman, 1994; Plendl et al., 1999). It is also possible that the growth factors that have been investigated in this and previous studies have had an indirect or paracrine effect on the ORNs. These issues can be examined for the first time using the clonal cell lines.

Functional responses and odorant receptor expression

Another application for these clonal cell lines relates to the control of odorant receptor expression. After stimulation with odorants, increases in the intracellular calcium concentration can be measured in the clonal cell line 3NA12. This calcium rise is presumably a result of an elevation of cAMP concentration,

which, in turn, opens the calcium-permeable, olfactory cyclic nucleotide-gated channels (Kurahashi and Shibuya, 1990; Leinders-Zufall et al., 1998). This suggests that some level of endogenous odorant receptor expression occurs in 3NA12 cells and that the cells can transduce odorant–receptor interactions. This indicates that the signal transduction machinery is not only present in these cells but is also functionally intact.

Both the percentage of 3NA12 cells responding to odors and the profiles of the responses in individual cells suggest that multiple odorant receptors may be expressed in the 3NA12 clonal cell line. Four individual, structurally diverse odorants were applied to 3NA12 cells, and functional responses to each odorant were observed in consistent, low percentages of cells. Among responsive cells, 90% of cells were stimulated by a single odorant, whereas 10% of cells responded to two odorants. The sum of the frequency of these responses was similar to the frequency of responses to a mixture of the same odorants in the primary cultures of dissociated rat ORNs. Because it is expected that the odorant receptors expressed by primary cultures of ORNs reflect a cross section of the total olfactory receptor population, our results suggest that a relatively broad spectrum of odorant receptors may be expressed by 3NA12 cells. The same conclusion can be derived from the response profiles of the cells. Responses were observed to each of the structurally diverse odorants tested, and the most direct explanation for this observation is that the cells in the 3NA12 cell line express different odorant receptors. Consequently, it can be proposed that the control of receptor expression in 3NA12 cells is dependent on extrinsic stimuli and is not determined entirely at a genetic level, as might be expected of clonal cells. This hypothesis can now be tested using the model system we have developed.

The low frequency of responses of the 3NA12 clone may also make these cells ideal for heterologous odorant receptor expression. In the past, receptor expression studies have been hampered by the lack of satisfactory heterologous expression systems in which odorant receptor proteins can be efficiently translocated to the plasma membrane. Because the 3NA12 cells are capable of functional responses and retain other differentiated features of olfactory receptor neurons, they are likely to be able to target exogenous olfactory receptors to the plasma membrane effectively and to signal odorant-induced receptor activation.

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