States of Developmental Commitment of a Mouse Embryonal Carcinoma Cell Line Differentiating along a Neural Pathway

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Abstract. The embryonal carcinoma cell line PCC7-S-AzaR$_1$ (clone 1009) has been shown to differentiate in the presence of all-*trans* retinoic acid and dibutyryl cAMP into cells of predominantly neural properties (Paulin, D., H. Jakob, F. Jacob, K. Weber, and M. Osborn. 1982. *Differentiation.* 22:90–99). By analyzing the marker expression of derivatives in further detail, we characterized the two major cell phenotypes as neuron- and fibroblast-like and the two minor ones as astroglia- and endothelial-like.

The stability of developmental commitment of clone 1009 was tested by recloning. The isolated subclones exhibited different patterns of chemically induced derivatives, with some of them (denoted N-clones) producing only a single (neuronal) cell type. As shown by long-term cultures in the absence of retinoic acid, the properties of isolated subclones remained essentially stable.

In contrast to the clones producing neuron-like and other derivatives upon induced differentiation, the (exclusively neuronal) derivatives of N-clones detached and died within a few days in culture. If maintained in the presence of other neural cell types, however, their survival was dramatically extended indicating a requirement for specific interactions with other cells of the same tissue.

The patterns of derivatives obtained from N-clones depended on the chemical nature of the substrate on which they were grown. Thus, when seeded on laminin-coated surfaces before induced differentiation, N-clones developed not only to neuron-like derivatives but rather to the same four derivatives observed with the original cell pool. These and further results suggest a common cell lineage of the identified phenotypes.

The isolated subclones of uninduced cells probably represent different states of commitment within the same developmental pathway. Their stability offers the opportunity to analyze the nature of cellular commitment on the cellular, molecular, and genetic levels. This makes the family of clones derived from PCC7-S-AzaR$_1$ (clone 1009) cells an advantageous in vitro model of mammalian brain early ontogenesis.

EMBRYONAL carcinoma (EC)[1] cell lines are well-established models for the study of cellular commitment, differentiation, and ontogenesis in mammalian systems (18, 24, 38, 53, 58). Concomitant with their increased application, however, the limits of these model systems have also been recognized: most EC cells show a predisposition to spontaneous differentiation (38, 39, 41), and their stem cell and differentiated states often possess only limited stability (20, 41, 61). Furthermore, the differentiation patterns of many EC cell lines are rather complex, in that they contain cell phenotypes corresponding to two or even all three germ layers of the developing embryo (27, 33, 55) in addition to extraembryonal tissue. As an example, F9 cells were shown to develop into derivatives with the characteristics of parental endoderm, visceral endoderm, adipocytes, neurons, and fibroblasts (33).

With the aim of developing an in vitro model of mammalian brain early ontogenesis for molecular studies, we have investigated the characteristics and stability of cellular states of the embryonal carcinoma cell line PCC7-S-Azar$_1$ (clone 1009) (1009) (13, 48, 49). From previous studies (14, 48), this EC cell line can be induced by all-*trans* retinoic acid (RA) to differentiate into derivatives of neural characteristics. Using a library of extracellular markers in addition to the intracellular markers previously used (48), we have been able to identify all four cell types developing under our conditions of chemical induction.

As expression of embryonic cell markers (in the absence of RA) was heterogeneous, we subcloned the original cell pool. We established several daughter clones differing from each other and from their parent cells with respect to their expression of embryonic markers and their potential to differentiate into the four identified cell types. In particular, we also obtained clones that in addition to homogeneously expressing all stem cell markers applied, were limited to differentiation into a single (neuron-like) cell type. These neuron-like derivatives were indistinguishable in cell mor-

1. *Abbreviations used in this paper*: dbcAMP, dibutyryl AMP; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; NF, neurofilament; pAs, polyclonal antiserum; PBS, phosphate-buffered saline; RA, all-*trans* retinoic acid; Rh, rhodamine; SSEA, stage-specific embryonic antigen; 1009, PCC7-S-AzaR$_1$ (clone 1009); TR, Texas red.

phology and marker expression from those obtained from our original pool of cells, except that they did not survive for long in culture. Their survival was dramatically extended, however, when they were cocultured with other brain-specific cells. Further studies indicated that the ontogenetic pathway of chemically induced derivatives (i.e., branching and maturation) depended on the presence of specific interactions with other cells of the same lineage, or factors secreted by them.

Our results suggest the existence of different states of commitment before chemical induction of differentiation. Cells of the particular state can be isolated by cloning procedures, and to a limited extent can be interconverted by variation of experimental conditions. From their limited phenotypic options, the marker expression of chemically induced derivatives, and their interdependence, the cells of clone 1009 and its subclones resemble cells of neuroectodermal origin.

Materials and Methods

EC Cell Line and Culture Conditions

Cell line PCC7-S-AzaR$_1$ (clone 1009) was kindly provided by Dr. H. Jakob, Institut Pasteur (Paris, France). It is an 8-azaguanine-resistant clone of the original cell line PCC7-S established from a spontaneous teratocarcinoma of a male recombinant inbred mouse (129 × B6) (13). In vivo differentiation (by injection into mice of hybrid stem 129 × B6) gave rise to well-differentiated tumors (13), suggesting that the cells of clone 1009 indeed are stem cells of teratocarcinomas. On the basis of chromosome number and banding pattern, the karyotype of the cell line appeared normal (13).

Cells were maintained and propagated in plastic tissue culture flasks (Greiner, Nürtingen, FRG) in DME (Flow Laboratories, Meckenheim, FRG) supplemented with 15% FCS (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in humidified air/10% CO_2. Cells were kept in exponential growth by seeding them at a density of 0.5–1.0 × 10^4 cells/cm^2 and replating every 2–3 d. At most, 40 passages were permitted, after which the cells were replaced by freshly thawed samples from standard stock stored under liquid nitrogen. Daughter clones of the original pool of cells were cultured (and differentiated, see below) at identical experimental conditions. For long-term continuous cultures, the same protocol was followed throughout the period of observation (up to 6 mo).

Cells were routinely tested for contamination by mycoplasms using staining with bisbenzimide H 33258 fluorochrome (Calbiochem-Behring Corp., Frankfurt, FRG).

Induction of Differentiation

Cells were seeded at a density of 1.5 or 3 × 10^4 cells/cm^2 in plastic tissue culture flasks or on glass cover slips (for immunofluorescence studies). They were allowed to adhere overnight before differentiation was induced. At that time (d0), all-*trans* RA (Sigma Chemical Co., Munich, FRG) was added to the culture medium to a final concentration of 10^{-7} M. (10 μl of a stock solution of 1 mg/ml DMSO, stored at −70°C, was first mixed with 190 μl DMSO, and the solution was mixed with 1,800 μl of distilled H_2O.) After 1 d (d1), the culture medium was replaced by DME supplemented with 15% FCS, 10^{-7} M RA and 10^{-3} M dibutyryl cAMP (dbcAMP) (Boehringer-Mannheim Biochemicals). Cells were maintained under these conditions without medium change for the whole period of observation.

The course of differentiation was observed using an inverted microscope (model IM 35; Carl Zeiss, Inc., Oberkochen, FRG); photographs were taken using a Plan 40 objective with a large working distance.

Cell Cloning

1009 cells (or of subclones, see below) of low passage number were cultured for 2 d in 75-ml plastic flasks. To be used as conditioned medium, the culture medium was removed, centrifuged, filtered through a membrane filter of 0.45 μm pore size, and stored at 4°C. After rinsing the cells three times with PBS, they were harvested by trypsinization (0.025% trypsin (Boehringer-Mannheim Biochemicals) in PBS for 3 min at 37°C. After addition of FCS to a final concentration of 10% (to stop trypsinization), we dispersed the cells by gently passing them three times through the orifice of a hypodermic syringe (type no. 20; TSK, Tuttlingen, FRG). This procedure routinely yielded >90% viable cells with no cell aggregates present.

Cells were seeded onto 96-well flat-bottom microtiter plates at densities of 50, 100, and 200 cells/plate, and grown in a 1:1 mixture of fresh medium and conditioned medium prepared as described above. After several hours of culture, each well was inspected under the microscope and those containing a single adhered cell were marked. Marked wells were inspected every day, and the grown cells resuspended by gently passing them through the orifice of a tissue culture pipette once every week. 2–3 wk after seeding, the cells were transferred into larger wells (24- or 12-well plates). The cell clones obtained in this way were further expanded, divided into aliquots, and stored in liquid nitrogen.

Antisera and mAbs

Polyclonal antisera (pAs) or mAbs were obtained commercially or were kindly provided as gifts from other laboratories. As embryonic cell markers, we used (*a*) mouse mAb anti-SSEA-1 (dilution 1:5,000; donated by Dr. B. Knowles, The Wistar Institute) (56), (*b*) mouse mAb ECMA-7 (1:10; donated by Dr. A. Starzinski-Powitz; Institut für Genetik, University of Cologne) (29) and (*c*) mouse mAbs RB 11-1 and RB 11-2 (undiluted; Dr. M. F. Rajewsky, Institut für Zellbiologie, University of Essen) (52). As neuronal markers, (*a*) mouse mAbs (dilution 1:5) directed against the neurofilament (NF) proteins NF68, NF160, and NF200 (Boehringer-Mannheim Biochemicals) (10), (ii) rat mAB 324 (1:50) directed against the L1 cell adhesion molecule (Dr. M. Schachner, Institut für Neurobiologie, University of Heidelberg) (51), and mouse mAb RB 21-7 (undiluted; Dr. M. F. Rajewsky) (31) were used. A mouse mAb directed against glial fibrillary acidic protein (GFAP) (1:10; Boehringer-Mannheim Biochemicals) served as the marker for astroglial cells (4, 10). Cells were characterized as fibroblast like by (*a*) a mouse mAb directed against thymocyte alloantigen Thy 1.2 (1:500; Sebak, Aidenbach, FRG) (42, 50), and (*b*) by rabbit pAs directed against the extracellular matrix proteins fibronectin (1:500; Calbiochem-Behring Corp.) (42) and laminin (1:50; Medac, Hamburg, FRG) (32). (Note that neuron- and astroglia-like cells of our system do not express Thy 1.2, so that discrimination of fibroblast-like cells in regard to the other cell types is unambiguous.) The mouse mAb anti-MESA-1 (1:10; Dr. C. Goridis, Department of Biology, University of Marseille) served as the marker for endothelial cells (16).

Other established markers used in this study were mouse mAb A2B5 (undiluted; hybridoma received from American Tissue Type Collection, Rockville, MD), which was directed against a ganglioside expressed on neurons and immature oligodendroglial and astroblasts of retina and cerebellum (3, 12); rat mAb H28 (dilution 1:5; Dr. C. Goridis), directed against neural cell adhesion molecule N-CAM (15); rat mAb 336 (1:25; Dr. M. Schachner, Heidelberg), directed against the carbohydrate epitope L2 of cell adhesion molecules (34); mouse mAb anti-GalC (1:10; Dr. B. Ranscht, University of St. Louis School of Medicine, St. Louis, MO) directed against galactocerebroside (GalC), a glycolipid on oligodendroglial cells (42, 50); mouse mAb anti-O1 (1:5; Dr. M. Schachner) and anti-O4 (1:50; Dr. M. Schachner) directed against cell surface antigens of oligodendroglial cells (57); goat pAs anti-vimentin (1:1,000; Dr. P. Jungblut, Max-Planck-Institut für Endokrinologie, Hannover, FRG, and Dr. P. Traub, Max-Planck-Institut für Zellbiologie, Ladenburg, FRG) marking mesenchymal cells (17, 47); rabbit pAs antidesmin (1:50; Medac), marking muscle cells (35); guinea pig pAs M9 (1:50; Dr. D. Breitkreutz, Deutsches Krebsforschungzentrum, Heidelberg) (6) and a rabbit pAs (1:50; Medac) directed against cytokeratin intermediate filament of epithelial cells; rat mAb TROMA (undiluted; Dr. A. Starzinski-Powitz), directed against Endo A cytokeratins of extraembryonal endoderm cells (23, 30). Reactivity of immune sera and antibodies was tested with the specific antigens (if available) and/or with established mouse cell lines and primary cultures (according to published protocols).

The following secondary antibodies were used: FITC-labeled rabbit anti-mouse Ig, rabbit anti-rat Ig·FITC, rabbit anti-goat Ig·FITC, rabbit anti-guinea pig Ig·FITC, and donkey anti-rabbit Ig·FITC (all 1:50 and from Dakopatts). In double immunofluorescence studies, the following additional antibodies were employed: Texas red-(TR) labeled goat anti-mouse IgM (1:50; Medac), goat anti-mouse IgG·TR (1:50, Dianova), goat anti-rat IgG·TR (1:80, Dianova), Rhodamine-labeled (Rh) rabbit anti-mouse Ig (1:50, Dakopatts), goat anti-mouse IgG·Rh (1:50, Dakopatts), rabbit anti-goat IgG·FITC and donkey anti-rabbit Ig·FITC (1:100, Amersham-Buchler, Braunschweig, FRG). All antibody dilutions were made with PBS supplemented with 10% FCS, 0.1% NaN_3.

Indirect Immunofluorescence Microscopy

Cell Surface and Extracellular Markers. Cells were plated on 12-mm-

diam round glass coverslips (Hecht) contained in wells of 24-well culture plates (Costar; Tecnomara). After the desired time of culture in the absence or presence of inducing agents, coverslips were removed, washed, and processed as follows: 50 μl of primary antibody solution was gently applied, and coverslips were incubated for 30 min at room temperature. After being dipped three times into PBS/0.2% FCS, 50 μl of secondary antibody solution was applied, and the coverslips were again incubated for 30 min, this time in the dark. After three washing cycles, cells were fixed in ethanol/acetic acid (95:5%, vol/vol) for 5 min at −20°C followed by three additional washing cycles. Coverslips were then mounted in Elvanol (1 g/ml)/glycerol (30% in PBS) supplemented with *p*-phenylene diamine dihydrochloride (1 mg/ml) to reduce fading of fluorescence (26).

Intracellular Markers. Immunostaining was performed by the same procedure as described above except that cell plasma membranes were permeabilized by fixation before application of first antibody. To stain cytokeratins, cells were fixed in methanol/acetone (1:3, vol/vol).

Stem Cell Markers on Detached Cells. EC cells were detached by gently rinsing with PBS/0.2% FCS. After counting the cells, 5×10^6 cells were incubated with 500 μl of stem cell marker antibody for 30 min at 4°C followed by washing with 10 ml PBS/0.2% FCS. After incubation with secondary antibody and another washing cycle, cells were fixed with 2% paraformaldehyde in PBS. Positive and negative cells were counted under the fluorescence microscope or by FACS performed as described in reference 31; the help of Klaus Lennarz at the Institut für Zellbiologie, University Essen, is gratefully acknowledged.

Double Immunolabeling of Cells. Immunostaining with the first set of antibodies was performed as described above for cell surface and extracellular markers. After three washing cycles, the second set of antibodies was applied without (cell surface markers) or with (intracellular markers) previous fixing of cells. Finally, cells were washed (fixed, if not yet done), and mounted as described above.

Immunofluorescence was analyzed with a fluorescence microscope (Carl Zeiss, Inc.) equipped with a HBO 100 mercury lamp, Zeiss fluorescence filter sets 00 for Texas red, 09 for FITC and 14 for Rhodamine excitation, and the following objectives: Neofluar-40-Ph2, Plan-Neofluar-40-Ph3, and Plan-Apo-63-Ph3. Immunofluorescence was recorded on films (HPS 400 ISO; Ilford) by means of a camera (OM 2N; Olympus, Kyoto, Japan) attached to the microscope.

Culture of Neuron-like Cells on Substrates

Proteins as Substrates. Cells were grown and differentiated on protein-coated surfaces. For this purpose, the culture vessels or glass coverslips were incubated for 1 h at 37°C with 250 μl/cm² of surface of solutions of 20 μg/ml laminin (Collaborative Research, Inc., Lexington, MA) or 64 μg/ml fibronectin (Boehringer-Mannheim Biochemicals) in PBS and subsequently washed three times with PBS.

Cells as Substrates. Differentiated cells with flat morphology were used as substrates for the culture of differentiating neurons. For this purpose, cells of the original pool were seeded in plastic flasks and differentiation was induced as described. At days 8–12 after induction, the neuron-like cells were removed by rinsing of the flasks while most of the flat cells remained attached. These were then detached by trypsinization, and washed and subcultured on glass coverslips at a density of 0.6×10^4 cells/cm². Occasional inspection by immunofluorescence microscopy ensured the absence of any neuron-like cells in these cultures.

Cells from clone N1 grown and differentiated on plastic surfaces were collected by trypsinization 3 d after induction, and were plated (8×10^4 cells/cm²) onto glass coverslips coated with flat cells obtained as described above and precultured for 1–2 d. Mixed-cell cultures were maintained in our usual culture medium supplemented with RA and dbcAMP. Adhesion and survival of developing neuron-like cells was followed for up to 5 d after plating using phase-contrast microscopy, or was analyzed 1–2 d after plating by immunofluorescence using neuron-specific antibodies.

Antibody-mediated Cell Lysis with Complement

Anti-SSEA-1 antibody-mediated lysis with Low·Tox·M rabbit complement (Camon, Wiesbaden, FRG) was used to test whether SSEA-1-negative cells existed in our cultures. 1×10^6 cells detached from confluent cultures (from our original pool of cells or from the appropriate daughter clone) were mixed with 250 μl of culture medium containing SSEA-1 antibody (1:250) and incubated for 45 min at 37°C. Cells were washed twice, transferred into 250 μl of culture medium containing rabbit complement (1:10), and incubated for 90 min at 37°C. After estimating the ratio of viable to dead cells using trypan blue staining, the complement solution was removed by centrifugation, and the cells were plated and cultured as usual until confluence was reached. This protocol was repeated (up to six times) and the ratio of viable to dead cells was determined each time.

Results

Properties of Original Cell Pool

The stock of cells used in the experiments described here was obtained from the first five passages of cultured 1009 cells after they were received from Dr. H. Jakob. At our usual culture conditions, i.e., in DME with 15% FCS and maintained at low density (by replating every 2 d), all cells of our original pool expressed the intermediate filament protein vimentin (47), whereas only 60–80% expressed the embryonal cell surface marker SSEA-1 (56).

When in vitro differentiation was induced by the addition of 10^{-7} M RA (d0) and 10^{-3} M dibutyryl cAMP (d1), cell morphology and marker expression changed in a well-defined, reproducible fashion (Fig. 1), giving rise to four major cell types (Table I). Type I cells have small cell bodies and extensive processes. They express the neuronal markers L1 (51), A2B5 (3, 12), and neurofilaments NF200, NF160, and NF68 (10). Expression of these markers varied with the time of culture in a fashion consistent with maturation of Type I cells. Type II cells have large cell bodies and flattened morphology. They express Thy1.2 alloantigen (42, 50) on their surface (no other cells of our system express this marker antigen), and deposit fibers of extracellular matrix containing fibronectin (42) and laminin (32). These properties characterize them as fibroblast like. Type III cells have similar morphology but are smaller than Type II cells. They often possess long extensions and express the astroglial cell marker GFAP (4, 10). This defines them as astroglia like. Type IV cells again are of flattened morphology; they express MESA-1, a marker for endothelial cells (16). In contrast, none of the other markers used (see Materials and Methods) were expressed by RA-induced derivatives of 1009 cells. In particular, neither epithelial cells (cytokeratin intermediate filaments [6]), nor extraembryonal endoderm cells (Endo A keratins [23, 30]), nor muscle cells (desmin [35]), nor oligodendrocytes (GalC [42, 50], O1, and O4 [57]) were observed in our cultures. (Based on the above definition of observed cell types, and if not stated otherwise, all subsequent characterizations of occurring cell types were based on the main markers listed in Table I. If not specifically characterized by their marker expression, we often refer to type II, III, and IV cells as "flat cells." As a general observation, cell proliferation drops soon after induction of differentiation, with only the fibroblast-like cells continuing to proliferate for a limited period of time. The derivatives therefore cannot be subcloned as has been described for other EC cell lines (43–45).

The characterized cell types appeared in chronological order in the course of the differentiation process (Fig. 2). 1 d after complete induction (d2), most if not all cells developed short extensions, and the first mature neurons (type I cells) appeared expressing L1, neurofilaments, and L2 antigens. They strongly increased in number until d4, when they began to express A2B5 (3, 12) and to form a network of processes. Beginning with d3, large flat cells appeared, forming by d6 confluent patches underneath and around the type I cell network. The majority of these flat cells belonged to type II (Thy1.2, fibroblast like). Concomitant with the formation of

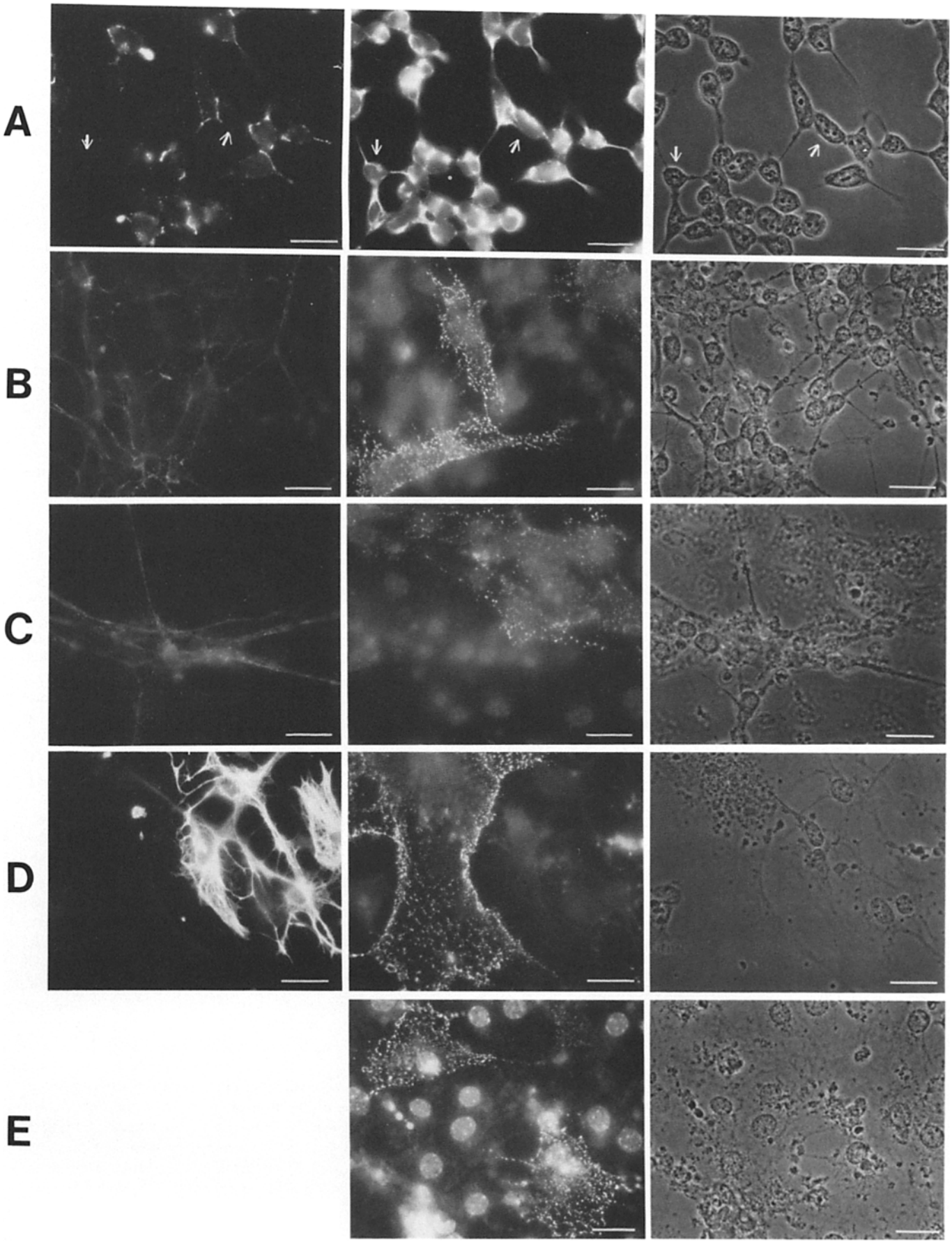
A
B
C
D
E

Table I. Cell Types, as Defined by their Marker Expression, of Original Cell Pool before and after Chemically Induced Differentiation

Cell type	Main marker	Additional marker	Relative distribution
			%
0, Stem cell-like	SSEA-1	Vimentin, ECMA 7; RB 11-1; RB 11-2; N-CAM	
I, Neuron-like	L1	NF200,160,68; A2B5; N-CAM; L2	40–50
II, Fibroblast-like	Thy1.2	Fibronectin; laminin; vimentin; N-CAM	30–40
III, Astroglia-like	GFAP	Vimentin; N-CAM; A2B5*; SSEA 1*	10
IV, Endothelia-like	MESA 1	Laminin; vimentin	<5

Relative distribution was determined by estimating at d7 after induction of differentiation the relative number of cells expressing their main marker.
* Expression by a subpopulation of cells.

first patches of confluent fibroblast-like cells (d5, d6), single cells with astroglial (type III, GFAP) and endothelial (type IV, MESA-1) properties appeared among the fibroblast-like cells. They slowly increased in number until d9. Type III (astroglia-like) cells were found in groups and, predominantly, in close proximity to aggregated neuron-like (type I) cells. Astroglia-like and, in particular, endothelial-like cells remained minor populations of RA-induced derivatives (Table I).

Based on this chronology of developmental events, five stages of the differentiation process may be defined (Fig. 2): stage 1 (d1, d2), at which cells already differ in marker expression and morphology from their stem cells but do not yet express markers of mature cells; stage 2 (d2–d4), at which neuron-like cells represent the predominant cell type. They initially exist as separate cells that later begin to form aggregates; stage 3, (d3–d6) at which network-like structures of neuron-like cells form, whereas fibroblast-like cells appear as the second major cell phenotype; stage 4 (d6–d8), at which neuron-like cells further aggregate, and astroglia-like and endothelial-like cells appear; and stage 5 (from d8), at which patches of neuron-like cells begin to detach and to lyse, with a few neuron-like cells remaining on top of a dense layer of flat cells.

Together, these results suggested a clear preference of our original pool of cells to differentiate, upon induction by RA (and dbcAMP), into phenotypes typical for neural tissue (see also Discussion).

Expression of Embryonic Cell Surface Markers by Original Cell Pool

As already reported at the beginning of this section, expression of stem cell marker SSEA-1 by our original pool of cells was heterogeneous. The same result was obtained by analysis of the expression of stem cell markers ECMA-7 (29), RB11-1 (52), and RB11-2 (52) by coverslip-adherent cells and by detached cells, using FACS for the latter studies (Fig. 3). As summarized in Table II, these markers were all expressed heterogeneously, independent of whether the test was performed immediately after plating or after several hours to 2 d in culture.

These data suggested the existence of different populations of cells in our original pool. To test whether this heterogeneity in stem cell marker expression was stably inheritable, we recloned the cells.

Expression of SSEA-1 Antigen by Clones of Original Cell Pool

Clones were obtained by limiting dilution of original cell pool, and nine of these clones (from two limiting dilution experiments) were selected for further studies. As exemplified in Fig. 3 by FACS analysis of SSEA-1 expression of clone O3 and as summarized in Table III, several of the selected clones were fully homogeneous with respect to expression of this embryonic antigen (clones N1, N2, O1–O3, and P2). We also obtained clones, however, of which only a fraction of cells expressed SSEA-1 antigen (N3, P1, P3). Interestingly, although our original pool of cells and clones N3, P1, and P3 contained cells negative in SSEA-1 expression, these could neither be cloned nor significantly be enriched by anti-SSEA-1 antibody-mediated lysis with complement.

Together, these data suggested stable levels of variance in undifferentiated cell properties. We therefore asked whether the different clones produced different patterns of induced derivatives, and whether there existed a correlation between these patterns and the level of embryonic marker expression.

Figure 1. Double immunofluorescence labeling and phase-contrast micrographs of original cell pool before and after chemical induction of differentiation by RA (10^{-7} M) and dbcAMP (10^{-3} M). Cells were plated on round coverslips contained in 24-well culture plates, cultured in the absence or presence of inducing agents, and processed for indirect fluoresence microscopy of antibody binding as described in Materials and Methods. Bar, 20 μm. Row *A* (d0): Expression of SSEA-1 antigen (*left*) and vimentin (*middle*) of the stem cells (*right*) in phase contrast. Arrows mark cells stained by anti-vimentin but not anti-SSEA-1 antibodies. Row *B* (d3 of differentiation process): Expression of L1 antigen by cells with long extensions (*left*) lying on top (different focus applied) of single large flat cells expressing Thy1.2 alloantigen (*middle*). (*Right*) Phase-contrast micrograph focused on the layer of cells with extensions. Row *C* (d6): Network of L1-expressing cells (*left*) with adjacent Thy1.2-expressing cells (*middle*). Note the aggregation of cell bodies of L1-expressing cells (*right*). Row *D* (d9): GFAP-positive cells (*left*) and Thy1.2-positive cells (*middle*) lying underneath aggregated neuron-like cells (not in focus). Note that Thy1.2-positive and GFAP-positive cells form separate patches. Row *E* (also at d9): MESA-1-positive cells (*middle*) lying underneath detaching neuron-like cells (*right*, phase contrast).

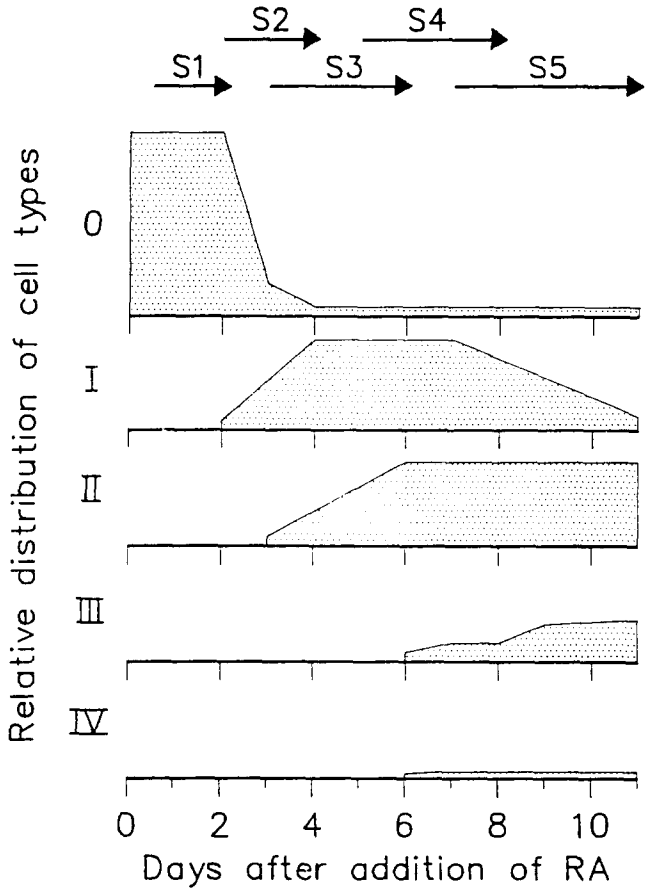

Figure 2. Schematic representation of the relative concentrations of identified cell types in the course of induced differentiation of original pool of cells. This scheme summarizes the results of a large number of experiments representatively shown in Fig. 1. 0–IV represent the different cell types; S1–S5 represents the different stages of developmental events. As detailed in Materials and Methods, RA was added at d0 and dbcAMP was added at d1 to the cultures of already adherent cells. To obtain a rough estimate of the relative concentration of cells of each phenotype, cells were immunolabeled and counted under the microscope. As cells not expressing embryonic or adult cell markers are not accounted for in this graph, the individual concentrations do not add up to 100%. The existence of type 0 cells at d3 and later is indicated by their outgrowth after removal of the inducing agents.

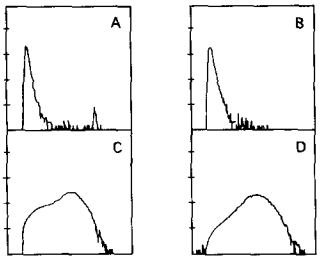

Figure 3. FACS analysis of SSEA-1 expression by original cell pool and by clone 03. Cells were detached by gently rinsing with PBS/0.2% FCS. They were then incubated with stem cell marker and secondary antibody, fixed, and subjected to FACS analysis (for details see Materials and Methods). A total of 2×10^4 cells were analyzed in each case, and the number of cells per channel of specific fluorescence intensity was plotted versus the fluorescence intensity (in arbitrary units). Controls (*A* and *B*) represent the distribution of fluorescence straylight of unstained cells. *C* and *D* represent the distribution of fluorescence of stained cells of 1009 cells (*C*) and clone 03 cells (*D*) after substraction of control values. The average fluorescence (in arbitrary units) was $m = 163$ and 260 for 1009 cells and 03 cells, respectively. At an equal channel position of maximal fluorescence, there is a higher level of negative cells in *C* (see pronounced shoulder at low fluorescence intensity). From quantitative analysis, 10–20% of 1009 cells did not express the SSEA-1 antigen, whereas all cells (within the limits of experimental error) of clone 03 expressed this marker.

Differentiation Patterns of Clones

When induced to in vitro differentiation, the clones produced rather different patterns of derivatives (summarized in Table III; Fig. 4). Although all clones gave rise to type I (neuron-like) cells, the presence of the other cell types varied both qualitatively and quantitatively for different clones. On the basis of their properties, the clones were divided into three groups (Table III).

Group N clones (N1–N3) differentiated almost exclusively into neuron-like (type I) cells. These rapidly (d2–d3) formed network structure but soon after detached and died. As shown in Fig. 4, for clone N1, the neuron-like derivatives attached only weakly to the plastic surface, even at early stages of the differentiation process (making the analysis of marker expression quite cumbersome). As determined at d3, N clone derivatives expressed L1 but not yet A2B5 or NF160 antigens. In a few experiments (see next section), single cells with morphological similarity to types II–IV cells were observed in addition. These could not be identified as specific cell type(s) as they lacked expression of typical cell markers. Before in vitro differentiation, all cells of clones N1 and N2 expressed SSEA-1 antigen, whereas clone N3 contained a few cells lacking expression of this embryonic marker.

Group O clones (O1–O3) also predominantly differentiated into neuron-like (type I) cells but also produced a small number of flat cells. Most of these could not be identified as specific cell types as they lacked the appropriate marker expression. In the majority of experiments, however, there existed at least a few cells weakly expressing fibroblast (type II) and astroglia (type III) markers, indicating that the marker-negative flat cells may have been immature cells. As an apparent consequence of their presence, neuronal (type I) cells in proximity to flat cells survived beyond d3, while the others detached and died. Before induced differentiation, all cells of group O clones expressed SSEA-1 antigen.

Group P clones (P1–P3) exhibited differentiation patterns resembling that of our original pool of cells, i.e., with sizable

Table II. Expression of Embryonic Markers by Original Cell Pool and Clone N1

Marker	Relative amount of labeled cells	
	Original pool	Clone N1
	%	
Vimentin	100	100
N-CAM	100	100
RB11-1	90	100
ECMA	80	100
RB11-2	80	100
SSEA-1	70	100

Cells were grown on glass coverslips and labeled with antibodies as described in Materials and Methods. Cells positive in indirect immunofluorescence were counted and related to the total number of cells seen in phase contrast.

Table III. Properties of the Original Pool of Cells and its Subclones

Stem cell-like cells		Chemically induced derivatives			
		Types of derivatives			
Clone	Expression of SSEA-1	I	II	III	Fate of Type I cells
	%				
Cell pool	80	+	+	+	Adhesion/survival
N1	100	+	−	−	Detachment/death
N2	100	+	−	−	Detachment/death
N3	95	+	−	−	Detachment/death
O1	100	+	−	(+)	Partial survival
O2	100	+	(+)	(+)	Partial survival
O3	100	+	(+)	(+)	Partial survival
P1	50	+	+	+	Adhesion/survival
P2	100	+	+	+	Adhesion/survival
P3	50	+	+	+	Adhesion/survival

Expression of phenotypic markers was determined in situ by indirect immunofluorescence at the day after induction optimal for detection of particular cell type. Types of derivatives: I, neuron like; II, fibroblast like; III, astroglia like. Plus and minus signs denote the presence or absence of particular cell type; plus sign in brackets denotes the presence in a majority of experiments of a limited number of cells of particular type. Type I derivates of original pool and P clones adhered and survived well (see Fig. 2), whereas derivatives of N clones detached and died within 2–3 d after induction. Survival of Type I derivatives of O clones depended on the presence and number of cells of flattened morphology.

numbers of flat cells expressing fibroblast and astroglial cell markers. Furthermore (Table III), only about half of the undifferentiated cells of clones P1 and P3 expressed SSEA-1 antigen (all cells of P2 were positive for SSEA-1).

These results established that the cells of our original pool were inheritable heterogeneous with respect to both embryonic marker expression and the RA-induced patterns of derivatives. However, the level of stem cell marker expression was not correlated with the types and numbers of derivatives obtained upon induced differentiation. Together, the different isolated clones seemed to be inheritably related to each other, i.e., they either derived from a common precursor cell or they are isomeric forms of a single cell type. To probe these alternatives further, the stability of marker expression of embryonic and derived cells of selected clones was tested.

Stability of Undifferentiated and Differentiated States of Clones N1 and O1

Clones N1 and O3 were kept in continuous culture for at least 50 passages (>4 mo). At given intervals, aliquots of cells were taken, analyzed for their expression of SSEA-1 antigen, and subjected to induced differentiation as described above. In the case of N1, we neither observed the appearance of flat cells in culture, nor an increased survival of neuron-like cells beyond d4. Simiarly in the case of O3, we again did not observe any changes in embryonic or derivated cell marker expression, or in the pattern of differentiation, indicating that the properties of cloned cells remain satisfactorily stable. Furthermore, seeding the cells at higher density (10^5 rather than 3×10^4 cells), did not affect the induced patterns of derivatives or their marker expression.

These data established that the heterogeneity of our original pool of cells, in terms of both undifferentiated and derivated cell properties, was indeed inheritable, defining the obtained clones as stable cell lines.

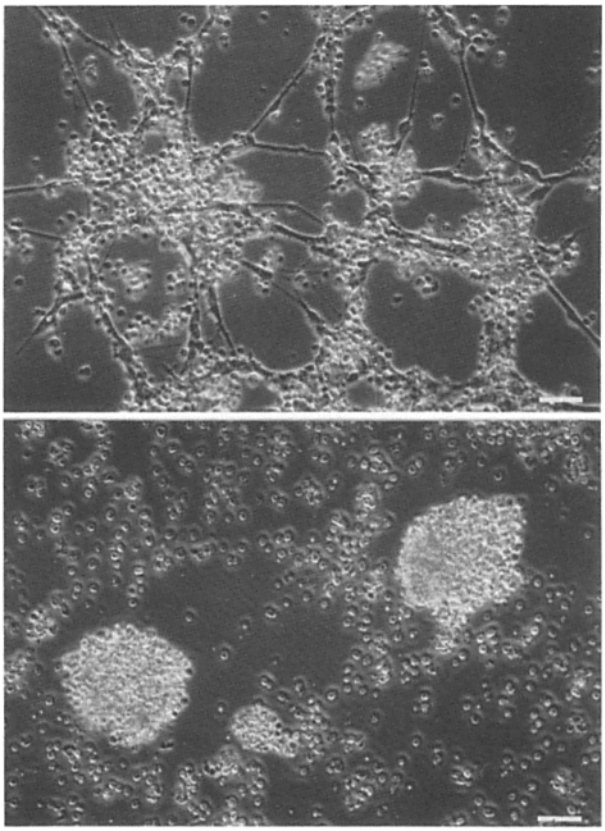

Figure 4. Phase-contrast micrographs of chemically induced derivatives of N1 cells. N1 cells were plated and induced to differentiate as usual. Although uninduced cells attached well to the plastic surface (indistinguishable from uninduced cells of parent pool, see Fig. 1 *A*), derivation is accompanied by increasing detachment of cells. (*Top*) A few cells have formed an extensive network structure while the majority of cells has already detached (d2). (*Bottom*) Cells have detached and died (d5). Bar, 50 μm.

Recloning of Clones

Because culturing the isolated clones over several months did not indicate instability of their undifferentiated and differentiated states (see above), the possibility remained that the heterogeneity in embryonic marker expression (original cell pool, clones N3, P1, and P3) and in RA-induced derivatives (original cell pool, clones O and P) had resulted from the process of cloning itself, i.e., from necessarily maintaining the cells for some time (14–18 generations) at lower than normal density. This hypothesis was tested by submitting several clones to recloning by limiting dilution. If the above hypothesis applied, each of the clones should give rise to a set of subclones falling into all three groups observed before (Table III). If the hypothesis did not apply, recloning should produce clones of identical properties as the parent ones. Three clones were tested in this way, N1, P2, and P3. The related data are summarized in Table IV.

Of the six subclones derived from N1, all six exclusively contained cells positive for SSEA-1. Furthermore, five of the subclones exhibited a closely similar, if not identical pattern

Table IV. Expression of SSEA-1 Antigen by Original Cell Pool and its Subclones, and by Clones N1, P2, and P3 and their Subclones

Parental clones			Subclones		
Clone	Expression of SSEA-1	Type of pattern	Number of subclones	Expression of SSEA-1	Type of pattern
	%			%	
			2	100	N
			1	95	N
Original pool	80	P	3	100	O
			1	100	P
			2	50	P
N1	100	N	5	100	N
			1	100	O
P2			2	100	O
	100	P	3	90–95	P
			2	70–90	P
P3	50	P	4	70–90	P
			2	30–50	P

of induced derivatives, indistinguishable from that of N1 parent clone. As described before for N1 (Tables II and III, Fig. 4), the cells differentiated exclusively into neuron-like (type I) derivatives; these rapidly formed network structures but soon after detached and died. Only one subclone of N1 produced a few singular flat cells and, thus, may be considered at the borderline between group N and group O clones.

In contrast, only the cells of two of the seven subclones of P2 all expressed SSEA-1 antigen. Three contained >90% SSEA-1-positive cells, the others contained much larger populations of SSEA-1-negative cells. The patterns of derivatives of the seven subclones, including their marker expression, were similar to that of the parent clone P2 which varied only quantitatively from that of our original pool of cells (Table III). Of the six subclones of P3, four exhibited a higher level of SSEA-1-positive cells and two a lower or equal level than the parent clone. Again, the patterns of derivatives were similar to that of the parent clone P3 and our original pool of cells (Table IV).

In summary, maintaining the cultures at low density (as in the initial phase of cell cloning) does not cause dramatic changes in either embryonic marker expression or the patterns of induced derivatives. However, as we observed minor (graded) changes under these conditions, it is necessary to maintaining the cells at relatively constant density.

From the results of recloning, (*a*) the level of stem cell marker expression and the patterns of derivatives resulting from induced differentiation again did not appear to be correlated; and (*b*) group N and group P clones appeared to represent the borderlines of a range of developmental options available to 1009 cells and their daughter clones.

Cell-Cell Interactions in the Course of RA-induced Differentiation

As one possibility for early cell death of derivatives of group N clones, neuron-like (type I) cells may require for their survival specific interactions with other cell types. To test this possibility, we harvested N1-cells of d3, seeded them onto layers of various other cell types, and tested their time of survival (see Materials and Methods). Our results follow.

(*a*) When seeded onto a confluent layer of flat cells obtained from our original pool of cells (stage 5, Fig. 2), N1 neuron-like derivatives attached and survived well, forming the usual network structures. Typically, the cells formed aggregates unevenly distributed over the culture dishes. (*b*) By immunofluorescence double-labeling experiments, which attempted to determine which types of flat cells were typically situated under and/or close to surviving N1 neuronal derivatives (Fig. 5), we observed predominantly GFAP-expressing (astroglia-like) cells and only seldom observed Thy1.2-positive cells. A preferential association of neuron-like and astroglia-like cells was also observed in cell cultures of our original pool of cells (Fig. 5). (*c*) So far, we did not succeed in mimicking the life-extending effect of cellular substrates by conditioned media of the same cells, or by seeding N1 neuron-like derivatives on laminin- or fibronectin-coated dishes. However, viable cells are not required for survival, as substrate cells fixed with 2% paraformaldehyde achieved the same effect as viable cells.

Although these results provided strong evidence in favor of specific interactions between the cell types of our model system, further experiments are required to fully expose their molecular basis.

Laminin Shifts the Pattern of Derivatives of Clone N1

Although laminin affected survival of N1 neuron-like derivatives only insignificantly, its effect on the differentiation patterns of N1 cells was dramatic. When N1 cells were grown in the absence of RA and dbcAMP on laminin-coated dishes, they expressed SSEA-1 antigen as before (100%). When induced by RA and dbcAMP to in vitro differentiation, however, not only neuron-like derivatives but, after the usual 2–3-d delay, large flat cells also appeared (Fig. 6). The neuron-like derivatives began to express L1-antigen at d2, and A2B5 and NF200 at d3. We did not observe any expression of NF160, not even at d8. The flat cells continued to increase in number until confluence was reached. The majority of flat cells expressed Thy1.2 alloantigen (type II cells), and some cells expressed GFAP (type III cells). With the reservation that we did not explicitly search for MESA-1-expressing (Type IV) cells, the overall pattern of derivates and the stages of differentiation were like those of our original pool of cells and group P clones. In particular, Type I (neuron-like) cells survived beyond d10 under these conditions.

These results suggest that the developmental options (level of commitment) of N1 cells (and by inference also of the other clones studied here) may sensitively depend on factors present in the culture medium and/or produced by other cells of the same lineage.

Discussion

The data presented here suggest that 1009 EC cells and their subclones represent a suitable model for mammalian brain early ontogenesis. This conclusion is based on the tissue specificity of cell phenotypes developing upon exposure to RA and dbcAMP, the time course and interdependence of developmental events, and their susceptibility to factors known to affect lineage branching and cell maturation.

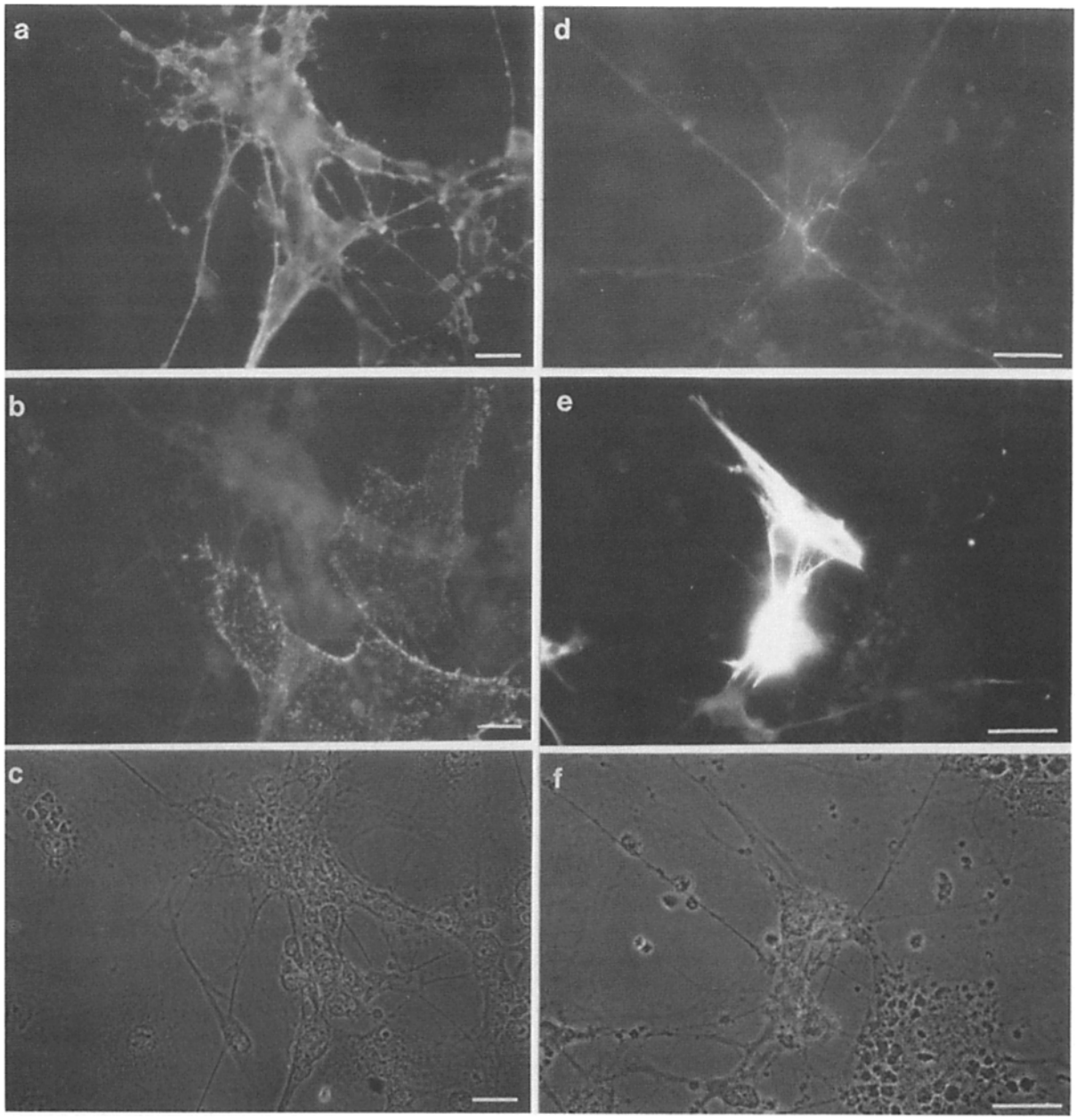

Figure 5. Interaction of neuron-like derivatives with flat cells. Left row (*a*, *b*, and *c*): N1 cells induced to chemical differentiation were harvested at d3 and seeded onto a layer of flat cells obtained at d9 from original cell pool (see Materials and Methods). N1 derivatives adhered well under these conditions, neurites grew out, and typical network structures were formed (*a* and *c*). The neuron-like cells expressed typical neuronal markers, e.g. A2B5 antigen (*a*). The flat cells positioned underneath the neuronal cell bodies typically expressed astroglial markers, whereas the Thy1.2-expressing cells were confined predominantly to other areas of culture dishes (*b*). (*c*) Phase-contrast micrograph of corresponding double immunofluorescence micrographs. Bar, 20 μm. Right row (*d*, *e*, and *f*): Double immunofluorescence of a culture of original pool of cells at d8 of the chemically induced differentiation process. For experimental conditions, see legend to Fig. 1. The neuron-like derivatives (characterized by L1-expression [*d*]) sat on top of flat cells expressing the astroglial marker GFAP (*e*). (*f*) Phase-contrast micrograph. Bar, 20 μm.

Tissue Specificity of Induced Derivatives

Previous in vivo and in vitro studies of PCC7-S EC cells have established their pluripotency (13). Such "uncommitted" EC cells have a tendency for spontaneous differentiation (38, 41) but, depending on the presence of specific drugs, may also be induced to differentiate along a limited number of developmental pathways (38, 40, 49, 58). In particular, the cell line PCC7-S-AzaR$_1$ (clone 1009) has been reported to develop upon exposure to RA and dbcAMP along a neural pathway (48). Using antibodies directed against intermediate filament proteins, Paulin et al. (48) identified neuron- and astroglia-like cells as major derivatives. Our studies expand these findings by identifying by means of established cell surface and intracellular markers all four cell types developing at these conditions in vitro. We identified neuron- and fibroblast-like cells as the two dominant populations and astroglia-

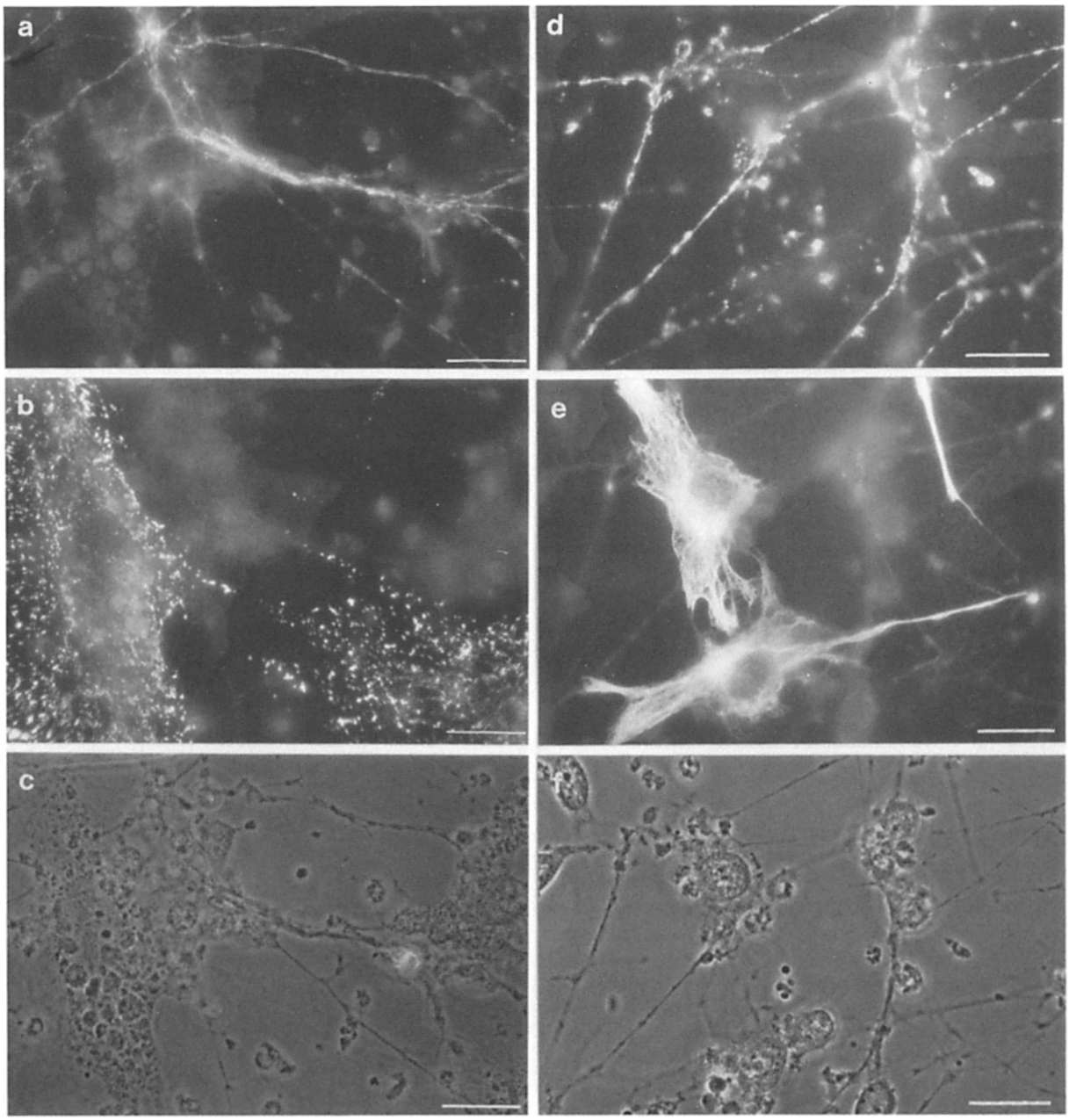

Figure 6. Morphology and marker expression of chemically induced N1 cells grown on laminin-coated surfaces. N1 cells were grown and differentiated on coverslips coated with laminin (5 $\mu g/cm^2$ surface). For details see Materials and Methods. Double immunofluorescence at d10 of differentiation of cultured cells expressing L1 (*a* and *d*), Thy1.2 (*b*), and GFAP (*e*). *c* and *f* are the respective phase-contrast micrographs. Thy1.2-expressing cells were typically found in areas devoid of cell bodies of L1-expressing cells (see also Fig. 4). Bar, 20 μm.

and endothelia-like cells as the two minor cell populations of RA-induced derivatives (Figs. 1 and 2 and Table I).

The developmental origin in vivo of neuronal and astroglial cells is established; they are derived from the neuroectoderm formed from the ectodermal germ layer as the product of neurulation. The developmental origin of the other two cell types is less clear. The mesodermal germ layer is the best known origin of fibroblasts and endothelial cells. It is now accepted, however, that certain types of mesenchymal cells (designated "ectomesenchymal") develop from the ectodermal germ layer, in particular the neuroectoderm of the neural crest (28, 36). The observed development restriction of 1009 cells compared with other EC cell lines may be the result of their neuroectodermal origin. The same descent has been suggested for P19 EC cells (37).

Independent of whether the four cell phenotypes of our model system are of common developmental descent, they are typical constituents of brain tissue. They are observed in primary cultures of various types of brain tissue (42, 50, 52, 54) and in sections of whole brain of various developmental stages (25, 52). It is therefore suggestive that the fibroblast- and endothelia-like derivatives observed in our model system are "neurofibroblasts" and "neuroendothelial" cells, respectively.

The tissue specificity of its RA-induced derivatives distinguishes the 1009 family of clones from most other EC cells (22, 38, 40, 41, 58, 61). Clone 1009 bears some resemblance with P19 EC cells which are known to differentiate upon induction by RA into predominantly neuron-, astroglia-, and fibroblast-like cells, whereas induction by DMSO produces derivatives of extraembryonic endodermal properties and muscle cells (40). As a significant difference, however, derivatives of the 1009 family of cells do not proliferate indefinitely, as has been reported for some RA-induced derivatives of P19, PC13, and F9 cells (43–45). This again is consistent with a neuroectodermal origin of 1009 cells.

In conclusion, at the used conditions of culture and of induction of differentiation, the cells of the 1009 family of clones may be considered operationally as committed to differentiating along a single (neural) pathway (see also below).

Stability of Committed State(s)

To be a suitable model for early neural development, stable commitment of cells is a basic requirement. Prompted by the known tendency of parent cell line PCC7-S to spontaneously differentiate (13), by the heterogeneous expression of embryonic cell markers by our original cell pool (Fig. 3, Table II), and by our identification of derivatives not necessarily originating from the ectodermal germ layer (see above), we submitted the original pool of cells to subcloning. These studies confirmed the heterogeneity of this cell pool. Long-term cultures and repeated recloning of subclones established, however, that heterogeneity develops only very slowly so that for all practical purposes subclones may be considered to be true cell lines. Furthermore, the heterogeneity in marker expression is not dramatically increased by the conditions of cloning itself. A remaining level of spontaneous variation in commitment may be a natural property, rather than a culture artifact, of embryonic cells. Thus, single cells from the neural crest of quail embryos of the 9–14 somites stage were found to possess a wide range of developmental capacity ranging from neuronal commitment to pluripotent capacity (2). Similarly, when single precursor cells from newborn rat retinas were marked by means of retrovirus-mediated gene transfer, segregation in diverse cell types was observed (60).

Embryonic Marker Expression and Level of Commitment

As summarized in Table III, a sizable number of isolated clones was homogeneous, others were heterogeneous with respect to the expression of embryonic marker SSEA-1. When the patterns of induced derivatives of these clones were analyzed, no direct correlation between the level of embryonic marker expression and the patterns of derivatives was observed (Table III). A high variance in the proportion of SSEA-1-expressing cells without consequences for their developmental potential (7, 19), and the isolation of cellular variants with altered responses to RA (61), have previously been reported for other EC cell lines. We conclude that the observed variance in SSEA-1 expression is unrelated to the level of commitment of these cells.

Differentiation Patterns of Variant Clones

As summarized in Table III, three types of differentiation patterns were observed for the isolated subclones; N clones, differentiating exclusively into neuron-like cells, O clones, differentiating predominantly into neuron-like cells with a minor population of flat cells present; and P clones, producing patterns of neuron-like cells and flat cells similar to that of our original pool of cells. Although these properties remained stable over many passages, further subcloning experiments (Table III) already suggested a tendency to limited interconversion between the three types of developmental options. The experiments with N cells seeded on laminin-coated surfaces before induced differentiation clearly established that the isolated clones do not differ *per se* in their developmental potentials. At these conditions, the full developmental capacity of original cell pool was recovered (Fig. 6, see also below). Consequently, the isolated clones do not represent genetic mutants as was suggested for the cloned variants of a different EC cell line (61).

Differentiation on Laminin-coated Surfaces

The simple differentiation patterns of N clones (only neuron-like derivatives) offers an excellent opportunity to probe whether factors exist capable of increasing the rate of interconversion of N cells into O or P cells, or shifting the state of commitment even further. As previously reported for C17-S1 1003 EC cells (8) and demonstrated for our system in Fig. 6, such factors indeed exist. Growing N cells on laminin-coated surfaces leads to a shift in their state of commitment, as they then differentiate, upon induction by RA, into the same four derivatives obtained from P clones and original pool of cells (Fig. 6). This shift in commitment affected neither the morphology nor the expression of embryonic markers of uninduced cells.

Extracellular matrix proteins have long been known to affect the differentiated state of cells (9, 21). Our findings suggest a modulation of the level of commitment of uninduced N cells rather than a shift to a different developmental pathway.

Chronology of Developmental Events

The existence of cellular variants differing in their patterns of RA-induced derivatives, and the laminin-induced shift in the level of commitment of uninduced N cells may both be related to the chronology of RA-induced developmental events: (*a*) as shown in Fig. 2, the induced derivatives of original cell pool appear in chronological order beginning with neuron-like cells. (*b*) Our cloning experiments yielded subclones that either exclusively differentiated into neuron-like cells (N clones) or into neuron-like and other cell types (O and P clones), but yielded no clones exclusively differentiating into any of the other three cell types. These findings may be rationalized by assuming that N cells have already passed the branching decision of commitment to becoming either flat cells or neuron-like cells. The effect of laminin may be a reversion of this branching decision on the level of gene regulation. Alternatively, laminin may simply stabilize the stem cell state (temporal protection from induction by RA) so that a remaining population of stem cells can later differentiate into nonneuronal derivatives.

The chronology of developmental events observed in this model system (e.g., for P cells) resembles those of rat brain ontogenesis in vivo and in vitro (1). Thus, concomitant with

the establishment of cultures of rat brain cells of embryonic day 10, neurons were observed, followed one day later by the appearance of fibroblasts. The latter increased in number, forming at d4–5 a confluent monolayer. Beginning at d5–6, astroglial cells were observed in small groups (1), just like in cultures of RA-induced 1009 cells and their P subclones. From these results, the chronology of developmental events apparently is not adversely affected by our culture conditions.

Interdependence of Cell Types

As shown in Figs. 1, 5, and 6, neuron-like derivatives of stages 4 and 5 are predominantly found on top of a layer of flat cells, in preferential association with astroglia-like cells. If flat cells are missing, as in cultures of derivatives of N clones, the neuron-like derivatives soon detach and die (Fig. 4). In contrast, when seeded on top of a layer of flat cells obtained at d8 from original pool of cells, survival of neuron-like derivatives of N clones was dramatically increased. The neuron-like derivatives thus appear to have a specific requirement for one or more of the other cell types (or their secreted products). Note, however, that neurite outgrowth of N cell derivatives (and by inference of neuron-like derivatives of other clones) is independent of the presence in culture of other developing cell types.

As shown in Fig. 5, the neuron-like cells of our culture system appear to preferentially associate with astroglia-like cells. These results agree with previous findings suggesting that neurons of the central nervous system preferentially adhere to astroglial cells as compared to fibroblasts (46).

The Isolated Clonal Variants Represent Different States of Commitment within the Same Developmental Pathway

The collective evidence of this study strongly suggests that N, O, and P clones are not genetic mutants of 1009 cells but rather represent different states of commitment of the same cells. Although reasonably stable under controlled conditions, interconversion of committed states can be fostered by the presence of specific factors such as laminin. Limited reversal of the level of commitment has previously been described for invertebrate systems (11), and recently also for mammalian systems (5, 59). It thus appears to be a natural property of stem cells rather than a cell culture artifact. From the tissue-specific patterns of derivatives after induced differentiation, the lineage relationship of derivatives, the availability of subclones of different states of commitment, and because the isolated clones and their derivatives are sufficiently stable and reproducible, the described family of clonal variants represents an advantageous model of early brain ontogenesis.

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