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Directed establishment of rat brain cell lines with the phenotypic characteristics of type 1 astrocytes

(cell immortalization/glial fibrillary acidic protein/oncogene)

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ABSTRACT **Interest in obtaining cell lines for use in studies on the development and biochemistry of the central nervous system has motivated efforts to establish cells from primary brain cultures by the use of oncogene-transfer techniques. In previous reports, cell lines derived from astrocytes in this way have had immature or abnormal phenotypes. We have explored the possibility of specifically "targeting" expression of exogenous oncogenes to differentiated astrocytes by using the promoter of the gene encoding glial fibrillary acidic protein, which is expressed almost exclusively in such cells. We report here that cell lines displaying the phenotypic characteristics of type 1 astrocytes can be established reproducibly in this manner. Given the heterogeneity of primary cultures, the availability of clonal cell lines displaying characteristics of type 1 astrocytes should greatly facilitate our understanding of the biology of these cells.**

Astrocytes are the most abundant cellular type in the brain and have traditionally been implicated in a variety of maintenance functions within the central nervous system (CNS) (1). Recent evidence indicates that these cells are much more dynamic components than previously realized. In particular, they sustain specialized interactions with neurons through the production of specific cell-adhesion, extracellular matrix, or neurotrophic molecules (2); they express functional receptors to most peptide and nonpeptide neurotransmitters present in the CNS (3); they are the main source of prostaglandins in the brain (4); and they produce a variety of peptidic precursors and plasma proteins (5–9). Astrocytes appear to be functionally heterogeneous. Thus, astrocytes from different regions of the brain express different sets of receptors (10), differ in their ability to produce certain proteins (5, 11), and display region-specific differences in their ability to support the growth and differentiation of neurons in culture (12). Local heterogeneity is also known to exist, as astrocyte subpopulations having distinct characteristics have been identified within a single anatomical region (9, 13–15).

Primary (nonestablished) rodent astrocytes in short-term cultures have been considered to be valuable *in vitro* cellular models for the study of the functions of their *in vivo* counterparts (16). The so-called type 1 astrocytes, which are the numerically predominant and most robust cells in such cultures, have been of particular utility. These are differentiated cells defined by specific morphological and phenotypic marker criteria (17). Cultures of type 1 astrocytes can be partially purified from other cell types by mechanical and/or immunological techniques (18, 19). However, such cultures are still clearly heterogeneous (9, 13–15) and may not be the most useful model for investigations of functionally specialized astrocyte subsets. Unfortunately, primary rodent astrocytes do not proliferate enough in culture to permit single-cell cloning and expansion without prior establishment (20). Thus, understanding of glial cell biology would be greatly facilitated by the availability of clonal cell lines with characteristics that are representative of selected subpopulations of type 1 astrocytes.

The potential utility of cell lines derived from astrocytes for studies of CNS development and biochemistry has motivated efforts to establish such lines through the use of oncogene-transfer techniques (DNA transfections or retroviral oncogene transduction). While some "glia-like" cell lines have been obtained in this fashion (20–28), these displayed mostly immature or abnormal phenotypes; establishment of well-characterized type 1 astrocytes from rodent brain has not been described. These results are surprising, since differentiated astrocytes would have been abundant or even predominant in the starting cultures used in previous experiments. In light of these findings, we considered the possibility of specifically "targeting" expression of exogenous oncogenes in differentiated astrocytes by using the promoter from a gene that is expressed most strongly in such cells. Molecular cloning and characterization of the human *GFAP* gene have been achieved (29); this gene encodes glial fibrillary acidic protein (GFAP), which is expressed almost exclusively in differentiated astrocytes (30). We report here that we have established primary astrocytes in a "directed" fashion by transfecting a DNA construct containing the oncogenic early region of simian virus 40 (SV40) under the transcriptional control of the human *GFAP* promoter and that a high proportion of these cells have the differentiated phenotypic characteristics of type 1 astrocytes.

METHODS

Materials and Reagents. Anti-GFAP antiserum was obtained from Dakopatts (Santa Barbara, CA); anti-transferrin and anti-α_2-macroglobulin antisera, anti-GFAP monoclonal antibodies, and rhodamine- or fluorescein-labeled secondary antibodies from Boehringer Mannheim; anti-galactocerebroside from Chemicon; and anti-SV40 large tumor (T) antigen monoclonal antibody from Oncogene Sciences (Manhasset, NY). The A_2B_5-producing hybridoma was from the American Type Culture Collection, and the monoclonal anti-O4 antibody was a gift from M. Dubois-Dalcq (National Institute of Neurological Disorders and Stroke, Bethesda, MD). Plasmid p62-1a, containing the 360-base-pair cDNA for rat S-100β

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Abbreviations: CNS, central nervous system; GFAP, glial fibrillary acidic protein; GABA, γ-aminobutyric acid; SV40, simian virus 40.

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protein was a gift from N. Sueoka (University of Colorado, Boulder). Plasmid pSP64E, containing the 1070-base-pair cDNA for rat proenkephalin A was a gift from J. Douglass (Vollum Institute, Portland, OR).

Recombinant Plasmids. pGFA-SV-Tt contains the human *GFAP* regulatory sequence of pGfaCat-2 (31) upstream of the SV40 early genes [*Stu* I–*Bam*HI fragment (32)]. pPGK-neo contains the murine phosphoglycerate kinase gene promoter (33) driving the neomycin phosphotransferase II–poly(A) cassette of pMC1polyA (34) (obtained from Stratagene). A mutation present in the original pMC1polyA (35) was corrected in the construct. Plasmids were propagated and purified by standard methods (36).

Cell Culture. Primary cultures of type 1 astrocytes were derived from either cortex or diencephalon from 1-day-old rats (5). All cultures were maintained at 39°C in Dulbecco's modified Eagle's medium H21 supplemented with 10% fetal bovine serum and penicillin, streptomycin, and fungizone, in an atmosphere of 5% CO_2 and 95% O_2. Oligodendrocyte and type 2 astrocyte culture was adapted from published protocols (18, 37, 38).

Transfection of Astrocytes. Primary cultures containing predominantly type 1 rat astrocytes were transfected 3 days after the initial plating with 5 μg of pGFA-SV-Tt and 0.5 μg of pPGK-neo by a calcium phosphate protocol (36), and transfectants were selected with G418 [0.8 mg (crude weight)/ml]. Individual colonies were isolated and expanded.

Immunological Methods. Immunofluorescence microscopy, immunoprecipitation of [^{35}S]cysteine-radiolabeled secreted proteins, and Western blot analysis were performed using published protocols (39).

Uptake of [^{3}H]γ-Aminobutyric Acid ([^{3}H]GABA). Published procedures were employed (20, 40).

RESULTS

Morphological and Marker Characterization of Cell Lines. Sixteen cell clones were isolated by transfection of 3-day-old cultures of astrocytes with plasmids pGFA-SV-Tt and PGK-neo. Nine clones were derived from cortex (CTX) and seven clones from diencephalon (DI). All the isolated clones developed into robust cell lines and were passaged 15–20 times without showing any sign of cell crisis. Characterizations were performed on cells between their 10th and 14th passage.

Type 1 astrocytes can be defined by several criteria including polygonal cell morphology and constitutive expression of the astrocyte-specific intermediate filament GFAP (17, 30). The fibroblast-like morphology of the cells described here (Fig. 1) is typical of type 1 astrocytes. Most cells also had a flat and nonrefractile appearance, consistent with a nontransformed phenotype. More than 95% of nuclei from all cells contained SV40 T-antigen immunoreactivity, as assessed by immunostaining (Fig. 1). All but three cell lines displayed detectable GFAP immunoreactivity 2 days after reaching confluence; the relative proportions of positive cells varied between cell lines (Table 1). The staining was repeated twice with cells at different passages and similar proportions were observed. Similar results were obtained with two different anti-GFAP antibodies. We also examined two cell lines at low density and found that confluency was not a requirement for continued GFAP expression (Fig. 1).

The GFAP immunoreactivity present in the cell lines was further characterized by Western blot analysis. As was true in extracts of primary astrocytes, the strongest band migrated at the position (≈53 kDa) expected for GFAP (30) (Fig. 2). Identical results were obtained with both anti-GFAP antibodies. The intensity of the bands detected by Western blot analysis was generally in good agreement with the immunocytochemical results (Table 1).

Staining of cells in the CNS for GFAP is not unique to type 1 astrocytes. Type 2 astrocytes are also GFAP-positive but differ from type 1 astrocytes by their morphology and expression of the antigen recognized by the monoclonal antibody A_2B_5 (17). GFAP-positive precursors of the type 2 astrocytes have also been described that express the O4 epitope (41). Several GFAP-positive cell lines established from mouse brains have been reported to express galactocerebroside (23), an antigen expressed in mature oligodendrocytes (17). Neither O4 nor galactocerebroside is expressed in type 1 astrocytes (17). None of the 16 cell lines displayed any positive staining with A_2B_5, anti-O4, or anti-galactocerebroside antibodies (data not shown). As a control,

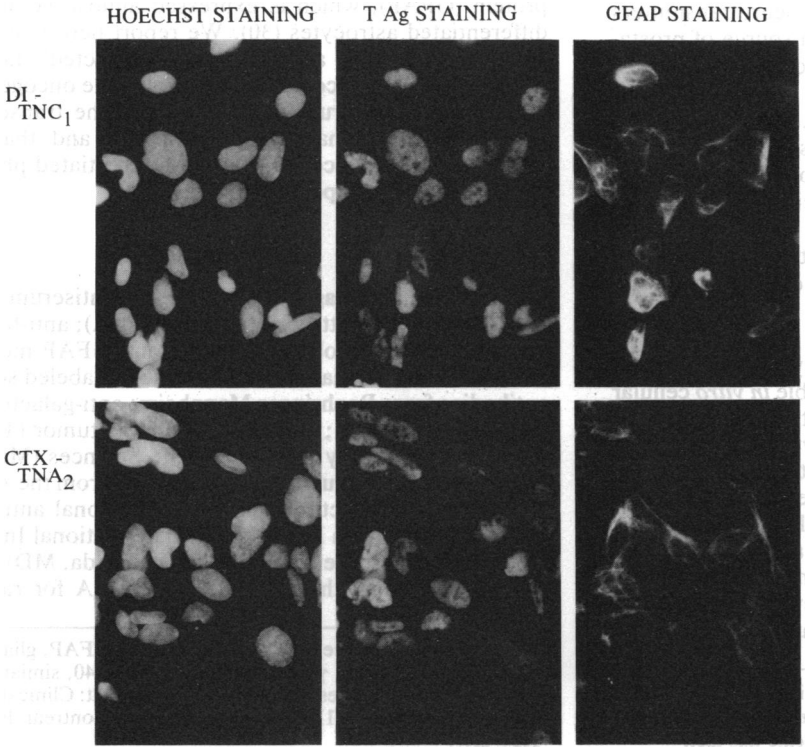

FIG. 1. Immunocytochemical characterization of established glial cells. Lines DI TNC_1 (*Upper*) and CTX TNA_2 (*Lower*) were seeded at low density and stained 48 hr later. The cells were triple-stained with Hoechst bisbenzimide 33258 (DNA stain) (*Left*) with monoclonal anti-SV40 T antigen (T Ag) and rhodamine-conjugated anti-mouse antibodies (*Center*), and with polyclonal anti-GFAP and fluorescein-conjugated anti-rabbit antibodies (*Right*). All the nuclei shown in the pictures contain T-antigen immunoreactivity. GFAP immunoreactivity was observed in most cells and was confined to cytoplasmic filaments.

Table 1. Characterization of cell lines

Cells	% GFAP+ cells	GFAP (Western)	S-100β mRNA (Northern)	GABA uptake	% GABA uptake with β-Ala	% GABA uptake with DABA
CTX TNA_2	80	+++	++	164 ± 11	8	106
CTX TNB_1	40	++++	ND	155 ± 16	10	115
CTX TNC_3	50	++	+++	375 ± 27	10	81
CTX TND_1	30	++	++	212 ± 35	9	72
CTX TND_3	40	±	++	248 ± 20	8	77
CTX TND_2	10	+	ND	234 ± 23	8	111
CTX TNC_2	20	−	+++	237 ± 36	11	99
CTX TNC_1	0	−	ND	261 ± 7	8	79
CTX TNA_1	0	−	ND	246 ± 37	10	82
DI TNC_1	80	+	+	118 ± 16	13	123
DI TNC_2	30	++	+++	118 ± 3	7	105
DI TND_1	30	+	+	34 ± 2	28	102
DI TNA_2	5	−	ND	282 ± 33	9	92
DI TNC_3	5	−	ND	125 ± 17	14	107
DI TNC_5	5	−	ND	73 ± 4	17	105
DI TNC_4	0	−	ND	120 ± 8	14	78
CTX ASTRO	95	++++	++	161 ± 10	14	76
DI ASTRO	95	++++	++	177 ± 23	18	102
NIE-115	0	ND	ND	9 ± 1	79	45

Characterization of nine cell lines established from cortical astrocytes (CTX) and seven cell lines established from diencephalon (DI). Primary cultures of astrocytes from either cortex or diencephalon are identified as ASTRO. NIE-115 is a mouse neuroblastoma cell line. In the second column, the approximate percentage of cells positively stained for GFAP is indicated. In the third and fourth columns, the relative intensities of the bands detected by Western blot analysis (GFAP; 53-kDa band) and Northern blot analysis (S-100β mRNA) are indicated; − signifies that no bands were detected; ND means not determined. The GABA uptake results are cpm × 10^{-3}/mg protein ± SE. In the last two columns, the GABA uptake in the presence of either 1 mM β-alanine or 1 mM diaminobutyric acid (DABA) is indicated as percentage of control.

we verified in parallel that these antibodies appropriately labeled primary cultures of type 2 astrocytes and/or oligodendrocytes (17).

Northern Analysis for S-100β Expression. RNA was extracted from eight cell lines and from primary astrocytes. S-100β mRNA was present in all cell lines, sometimes in greater abundance than in primary astrocytes (Fig. 3). No obvious correlation was observed between the abundance of S-100β mRNA and the amounts of GFAP expressed by each of these cell lines (Table 1).

Uptake of [^{3}H]GABA. Primary astrocytes have been reported to display high-affinity uptake of GABA that is sensitive to inhibition by β-alanine (20, 40). As seen in Table 1, most cell lines took up GABA [present in the culture medium at a low (30 nM) concentration] in amounts comparable to primary cultures of astrocytes; the uptake was inhibited by β-alanine, whereas L-2,4-diaminobutyric acid had no appreciable effect. In contrast, the uptake of GABA by the neuroblastoma cell line NIE-115 was inhibited more by diaminobutyric acid than by β-alanine.

Secretion of Marker Proteins. Recent reports indicate that primary cultures of astrocytes synthesize and secrete a host of specialized proteins (5, 7–9). The production of transferrin and α_2-macroglobulin was assessed by immunoprecipitation of culture medium from cells labeled briefly with [^{35}S]cysteine (Figs. 4 and 5). A single transferrin band of ≈76 kDa was

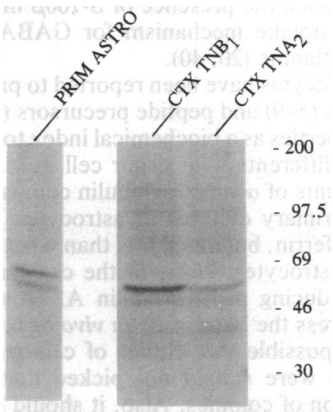

FIG. 2. GFAP Western blot analysis. Extracts from either primary cultures of astrocytes (PRIM ASTRO) or two of the transfected cell lines (CTX TNB_1 and CTX TNA_2) were analyzed (39). The strongest immunoreactive band in each sample migrated at ≈53 kDa. Positions of molecular size (kDa) markers are indicated.

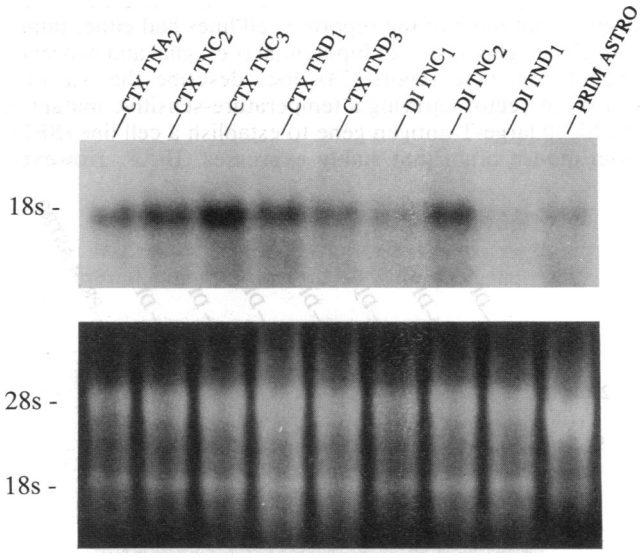

FIG. 3. S-100β mRNA Northern blot analysis. Samples (10 μg) of total RNA from eight representative cell lines and primary cultures of rat astrocytes were analyzed (36). (*Upper*) Autoradiogram of blot. (*Lower*) Ethidium bromide-stained gel. Migration positions of 18S and 28S rRNAs are indicated.

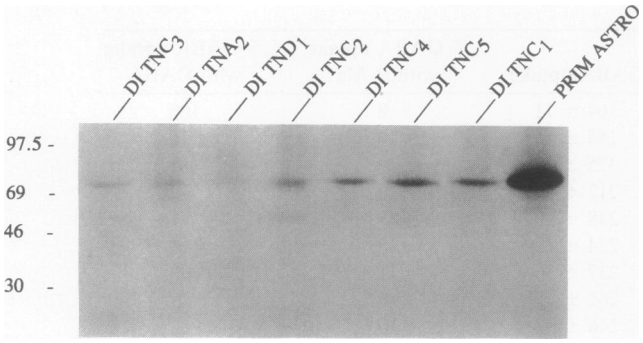

FIG. 4. Transferrin immunoprecipitation. Autoradiogram of [^{35}S]cysteine-radiolabeled protein (76-kDa band) secreted by eight representative cell lines and a primary culture of rat astrocytes. Positions of molecular size (kDa) markers are indicated.

immunoprecipitated from the incubation medium of all the cell lines, although the cell lines produced much less of this protein than 3-week-old primary cultures of astrocytes (Fig. 4). In contrast, all the cell lines produced α_2-macroglobulin in amounts similar to or higher than those found in the primary cultures. While the majority of the material migrated at ≈300 kDa, smaller degradation products were also detected (Fig. 5), in agreement with previous observations (9).

To determine whether the cell lines produced proenkephalin A, the cultures were incubated for 2 days in serum-free defined medium containing 1 mM dibutyryl cyclic AMP. Northern blot analysis failed to detect proenkephalin A mRNA from any of the cell lines, although it was present in 3-week-old primary cultures of astrocytes (data not shown).

DISCUSSION

The large-T-antigen gene from SV40 belongs to a group of genes that has the ability to establish the continuous proliferation of primary cells in culture (42–45). Glial-like cells can be established from primary rodent brain cultures by transfer of such genes; examples include the infection of primary cultures from rodent brains with SV40 (26) or with retroviral vectors containing the coding sequences for N- or c-*myc* (22, 24, 25, 27) or for SV40 large T antigen (21, 22, 28). Cells with some astroglial characteristics have been established in this fashion, but most of the reported cell lines had either immature glial ("glioblast") or bipotential (i.e., glial and neuronal) phenotypes. One report (23) does describe the use of a retroviral vector carrying a temperature-sensitive mutant of the SV40 large-T-antigen gene to establish a cell line (SF11) from mouse brain that stably expresses GFAP. However,

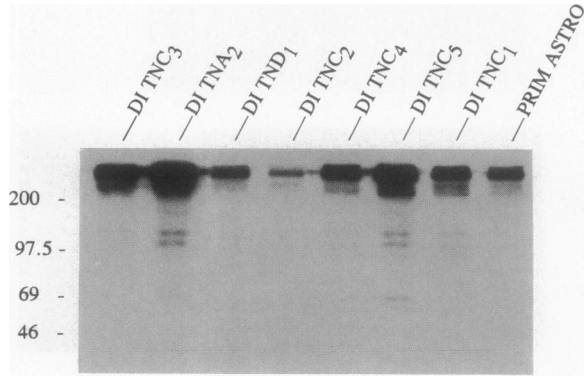

FIG. 5. α_2-Macroglobulin immunoprecipitation. Autoradiogram of [^{35}S]cysteine-radiolabeled protein shows 300-kDa major band secreted by eight representative cell lines and a primary culture of rat astrocytes. Positions of molecular size (kDa) markers are indicated.

isolation of the GFAP-expressing line was a rare event (1 out of 19), and the line itself was not examined for other characteristics of type 1 astrocytes.

While retroviral vectors can be a highly effective way to introduce genes into mammalian cells, their use is limited in that proviral DNA integration and gene transfer require dividing cells (46). This fact may explain why most of the glial cell lines reported to date have immature phenotypes despite the predominance of more mature (and presumably less proliferative) cells in the starting cultures. An alternative procedure for the establishment of cell lines is to transfect oncogene expression plasmids into cultured cells. Evrard *et al.* (20) used this method with constructs containing polyomavirus large-T-antigen gene and adenovirus E1A genes under transcriptional control of constitutive promoters to obtain cell lines with some astrocytic features. However, unlike type 1 astrocytes, these cells expressed GFAP immunoreactivity only after induction with dibutyryl cyclic AMP or fibroblast growth factor, and the cells did not take up GABA as avidly as primary astrocytes (20).

We reasoned that the likelihood of obtaining well-differentiated cell lines would be increased by driving oncogene expression with a promoter preferentially active in mature astrocytes. The *GFAP* promoter was selected for this purpose, as the gene is expressed abundantly and almost exclusively in mature astrocytes in the CNS (29). A complete genomic clone of the human *GFAP* gene has been isolated (29), and its 5′ portion confers appropriate transcriptional specificity in transiently transfected cells (31). In our construct, we placed this 5′ segment upstream of the oncogenic SV40 early-region sequences that encode the large and small T antigens.

The success of this approach for obtaining multiple cell lines with the characteristics of differentiated astrocytes is described in this report. Thus, a majority of the cell lines (13 out of 16) displayed some degree of immunoreactivity for GFAP in the absence of any special treatment or hormonal induction [which can sometimes induce GFAP expression in immature astrocytes where it is otherwise absent (20)]. All the cell lines were negative for galactocerebroside and for the antigens recognized by the monoclonal antibodies O4 and A_2B_5, indicating that these cells are most likely representative of type 1 (rather than type 2) astrocytes (17). In the lines having the highest proportions of GFAP-positive cells (CTX TNA_2 and DI TNC_1), expression of this marker was not found to be strictly dependent upon confluency. This behavior is in contrast to that of an astrocytic cell line described previously that was only GFAP-positive at high cell density (47). Other features that were consistent with the phenotype of type 1 astrocytes included the presence of S-100β mRNA (48) and a high-affinity uptake mechanism for GABA that was inhibitable by β-alanine (20, 40).

Cultured astrocytes have been reported to produce several plasma proteins (5–9) and peptide precursors (6, 14), and we used these properties as a biochemical index to further assess the degree of differentiation of our cell lines. All the lines produced amounts of α_2-macroglobulin comparable to those produced by primary cultures of astrocytes. They also all produced transferrin, but much less than what was observed with primary astrocytes. None of the cell lines was found capable of producing proenkephalin A. However, not all astrocytes express the latter gene *in vivo* or *in vitro* (14, 15); it is therefore possible that clones of cells expressing this particular gene were simply not picked during the initial random selection of colonies. Also, it should be noted that, for technical reasons, production of these proteins was assessed in 3-week-old primary cultures of astrocytes, whereas the cell lines were derived from 3-day-old cultures. Some biochemical "maturation" of astrocytes occurs in the course of the first few weeks in culture (4, 14, 49); for

example, there is an increase in the percentage of proenkephalin-producing astrocytes over this time period (14). Thus, while the cell lines presented in this work clearly have differentiated characteristics of type 1 astrocytes, they might be most representative of 3-day-old primary cells.

We have established multiple cell lines with the phenotypic characteristics of type 1 astrocytes, thus validating the approach we have employed. We expect that this approach of driving oncogene expression with promoters preferentially expressed in mature cells should be generally applicable for the efficient isolation of differentiated cell lines. In the particular case of astrocytes, it may be possible to isolate distinct subclasses of these cells that have been proposed to perform different roles in the CNS (5, 10, 11, 13, 15). The different phenotypic profiles displayed by our cell lines (Table 1) could in fact be a reflection of this proposed heterogeneity. Studies of such cell lines would greatly enhance our understanding of the contributions of astrocytes to brain functions.

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