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AF5, a CNS Cell Line Immortalized with an N-Terminal Fragment of SV40 Large T: Growth, Differentiation, Genetic Stability, and Gene Expression

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Central nervous system progenitor cells that are self-renewing in culture and also differentiate under controlled conditions are potentially useful for developmental studies and for cell-based therapies. We characterized growth and plasticity properties and gene expression in a rat mesencephalic cell line, AF5, that was immortalized with an N-terminal fragment of SV40 large T (T155g). For over 150 population doublings in culture, the growth rate of AF5 cells remained steady, the cells remained responsive to bFGF, and telomerase activity and telomere lengths were unchanged. While karyotype analyses revealed some chromosomal abnormalities, these were also unchanged over time; additionally, no mutations in p53 gene sequences were found, and wild-type p53 activation was normal. AF5 cells produced PDGF, TGFβ1, TGFβ2, and bFGF. Similar to primary progenitor cells, AF5 cells retained their plasticity in culture; they could be propagated in an undifferentiated state as "neurospheres" in serum-free media or as adherent cultures in serum-containing media, and they differentiated when allowed to become confluent. Adherent subconfluent actively growing cultures expressed a marker for immature neurons, nestin, while few cells expressed the mature neuronal cell marker βIII-tubulin. Confluent cultures ceased growing, developed differentiated morphologies, contained few or no nestin-expressing cells, and acquired βIII-tubulin expression. Global gene expression was examined using a 15,000 gene microarray, comparing exponential growth with and without bFGF stimulation, and the differentiated state. The AF5 cell line exhibited stable genetic and growth properties over extended periods of time, while retaining the ability to differentiate *in vitro*. These data suggest that the AF5 cell line may be useful as an *in vitro* model system for studies of neural differentiation.

Key Words: **SV40 large T; cell lines; immortalization; differentiation; microarray.**

INTRODUCTION

As an alternative to fetal tissue, central nervous system (CNS)-derived cell lines are used to study basic neurobiological processes and as potential cell-replacement therapies. Tumor-derived cell lines, such as rat PC12 (20), human SH-SY5Y (2, 37), Ntera (7, 29) and others, are routinely used as models for examining differentiation, neurite extension, signal transduction, apoptosis, and other molecular and cellular processes. Because tumor-derived lines are not ideal due to their many abnormalities, immortalized, nontumor cell lines continue to be developed for these purposes (reviewed in 19, 60, 61) and primary neural progenitor or "stem" cells are being used for generating neurons or neuron-like cells in culture (reviewed in 14–16, 33, 36, 38, 46, 58). Ultimately, genetically identical and homogeneous neuronal cell lines with defined characteristics, whether established from "stem-like" progenitor cells or from primary fetal tissue, may best serve the purposes of transplantation or as models for specific functions.

Cell lines can be established by transducing oncogenes into primary cell cultures (reviewed in 5, 61). Oncogenes commonly used to produce CNS cell lines include v-myc (43) and SV40 large T (LT) (4, 50). In some cases, however, the oncogenes used to create cell lines appear to interfere with expression of mature phenotypes and differentiation (10, reviewed in 4; also unpublished observations). Various remedies are being explored, such as site-specific recombination to excise the oncogene (59) or the use of inducible promoters (22), which can be manipulated allowing the cells to

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differentiate. Another approach involves modification of the oncogene itself. Temperature-sensitive mutants of LT, such as tsA58, have been used to this end (24); however, temperature-sensitivity of growth is variable, and loss of temperature-sensitivity by additional spontaneous mutations in the tsA58 gene has been reported (51).

As an alternative, we developed a truncated LT called T155, now called T155g (52), with reduced ability to interfere with molecular mechanisms that may effect expression of neuronal phenotypes and differentiation. Full-length LT consists of domains that bind to, interact with, or inactivate cellular proteins key to growth, differentiation, and maintenance, including p300, Hsc70, retinoblastoma, p53, and a number of additional proteins (1, 9, 28, 30, 40, 44, 45, 50). T155g lacks three-fourths of the C-terminal region, thus eliminating the p53-inactivating and DNA binding domains and the region containing the mutation for temperature-sensitivity in LT tsA58. Nevertheless, it has the capacity to immortalize cultured rat CNS cells and kidney cells (11, 52, 54). The AF5 cell line was established previously by immortalizing primary fetal rat mesencephalic tissue in culture using T155g. The T155g plasmid construct, the methods used for establishing the cell line, and initial characterization of the cell line were reported previously (52).

A major consideration in the generation of cell lines *in vitro* is the degree to which such cells resemble tumor cells. The rationale for developing T155g as an immortalizing agent was that, because the p53-inactivating domain was missing, cells immortalized with this peptide would preserve important p53 functions that provide protection against damage or tumor formation. Preservation of p53 function is desirable in cells slated for transplantation. Cells may develop the ability to grow in culture beyond their normal life span directly via the effects of the transduced oncogenes, or simply by selection pressure during prolonged maintenance *in vitro.* Therefore, one objective of the present study was to examine, in long-term culture, properties of these cells that are related to the process of immortalization. We found the AF5 cell line to be stable in long-term culture; p53 gene sequence, wild-type p53 activation, karyotype, and telomere maintenance were unchanged over long-term culture.

The growth properties of the AF5 cell line prompted further study, as these cells appear to retain plasticity similar to primary neural progenitor or stem-like cells. Neural progenitor cells can be grown in suspension as clusters of immature cells, called neurospheres, in serum-free media under the influence of growth factors such as epidermal growth factor and/or basic fibroblast growth factor (bFGF) (39, 47). Neural progenitors can then be induced to differentiate in culture by adding serum to the media, by coatings that promote attachment to the growth surface, or by manipulation of growth factors. In this study we demonstrated that, similar to primary neural progenitor cells, AF5 cells can be propagated as neurospheres and will also differentiate in serum-containing culture media when allowed to become confluent. We characterized the differentiation properties by immunocytochemistry and by gene expression profiling using a 15,000 gene cDNA microarray. The microarray compared cells growing with and without bFGF and in the differentiated condition. A summary of genes representative of specific cell types or functions that were expressed in all three conditions is presented, as well as a summary of genes that are either increased or decreased uniquely in each of the three conditions.

METHODS

Cell Cultures

Establishment of the AF5 rat mesencephalic cell line using T155g was described previously (52). Unless otherwise indicated, cultures were maintained in a 5% CO_2 incubator in medium consisting of Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12, 1:1, Gibco Life Technologies, Gaithersburg, MD), 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin G, 100 ug/ml streptomycin. Mesencephalic glia were cultured from primary embryonic day-14 rat fetuses derived from outbred Sprague–Dawley rats (Charles River Labs), as described previously. Animal studies were performed in accordance with the NIH Guide for the Care and Use of Animals.

Immunocytochemistry

Adherent cells in flasks or multiwell plates were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 7–10 min and then permeabilized with ethanol:acedic acid (95:5) for 1–2 min at −20°C or fixed and permeabilized in the same step with 100% ethanol. Following washes, nonspecific binding sites were blocked for at least 20 min with buffer containing 10% normal goat serum and 0.1% bovine serum albumin. Primary antibodies were diluted in blocking solution and applied to the cells overnight at 4°C. Secondary antibodies, diluted in 10% blocking solution in PBS, were applied to the cells for 1 h. Counterstaining of nuclei was performed using 4′,6-diamidine-2′-phenylindole (DAPI). The cells were prepared for fluorescence microscopy by covering with Slow Fade Light mounting solution or buffer (Molecular Probes, Eugene, OR), then viewed using a Zeiss inverted microscope with the appropriate filters. Images were captured using a Photometrics Cool Snap digital camera with IPLab software. Primary mouse monoclonal antibodies used were, anti-βIII-tubulin (diluted 1:1000, Promega Inc, Madison, WI), anti-rat tyrosine hydrox-

ylase (diluted 1:100, PharMingen, San Diego, CA), and anti-rat nestin (1:500, Chemicon Inc., Temecula, CA). A primary rabbit polyclonal antibody to glial acidic fibrillary protein (GFAP) was used diluted 1:200 (PharMingen). The fluorophore-labeled secondary antibodies were Alexa 594 goat anti-mouse IgG and Alexa 488 goat anti-rabbit IgG diluted 1:400 (Molecular Probes).

Growth Factor Production

To measure the concentrations of growth factors secreted into culture media, cells were grown in 25-cm^2 culture flasks to about 80% density in 3 ml of serum-free media (DMEM/F12 with 2 mM L-glutamine, 100 U/ml penicillin G, 100 ug/ml streptomycin). After 24 h, the media were collected and particulates removed by centrifugation. The supernatants were analyzed in 96-well plates for brain derived growth factor (BDNF), platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF β1), transforming growth factor β2 (TGF β2), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), and epidermal growth factor (EGF) by Quantikine enzyme immunoassay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Glia derived neurotrophic factor (GDNF), was measured by an R&D Systems ELISA reagent set according to the manufacture's recommendations as described by Johnston *et al.* (25). Optical density was determined in a plate reader set at 450 nm with wavelength correction set at 540 nm. All antibodies and recombinant GDNF were supplied by R&D Systems.

Long-Term Growth of AF5 Cells

AF5 cells were cultured with or without bFGF for 24 weeks in 25-cm^2 flasks and passaged each week by reseeding them at a density of 2×10^5 cells per flask. At week 17, sister cultures from each condition were switched to the opposite condition. At 2- to 3-week time intervals, the cells were counted for four consecutive days after passaging. Cells in live cultures were counted visually using a 20× lens on an inverted Nikon microscope (30–50 fields per flask). The number of days required for the cells to double in number (doubling time) was estimated from these cell counts by fitting the growth curve to the logarithmic function $y = a \log x - b$, where y = number of days, x = number of cells, and a and b are constants. Doubling time in days was obtained by multiplying the factor a by 0.301. The cumulative number of population doublings over time was derived from these calculations.

Karyotype Analyses

The chromosomes of AF5 early and late passage (over 50 population doublings) cultures and normal fetal rat mesencephalic primary cultures were analyzed by Diagnostic Cytogenetics (Seattle, WA). The primary mesencephalic cells were pooled from several fetuses and cultured for 8 days prior to analysis. Chromosomal comparisons were made with previously published analyses of *Rattus norvegicus* karyotype (8, 21, 41).

Telomerase Activity and Telomere Length Assay

Cells were harvested from early and late passage cultures (the same cultures used for the long-term growth experiment described above) and washed with PBS. Cell pellets were stored at −80°C and assayed at the same time after all samples were harvested. Telomerase activity was measured using a Telomeric Repeat Amplification Protocol according to the manufacturer's instructions (PharMingen). The control reactions were heat-treated lysate (85°C, 10 min) or no lysate. The PCR products were resolved by 12.5% nondenaturing polyacrylamide gel. The telomere repeats were viewed by staining the gel with SyBrGreen (Molecular Probes). The image was captured with an AlphaEase imaging system (Alpha Innotech Corp., San Leandro, CA.). Telomere length was measured using PharMingen Teloquant kit, following the manufacturer's instructions. Briefly, genomic DNA from early and late passage AF5 cultures was extracted (Promega Wizard genomic DNA extraction kit). The DNA (2 or 2.5 μg) was digested with a mixture of Hinf I and Rsa I restriction endonucleases. The resulting restriction fragments were resolved by electrophoresis on a 0.6% agarose gel and transferred onto a nylon membrane (Hybound-N+, Amersham, Piscataway, NJ). The membrane was hybridized with a biotinylated telomere probe provided in the kit, and bands were detected with a chemiluminescent method according to the manufacture's protocol. The membrane was exposed to X-ray film to visualize the bands.

p53 Gene Sequence Analyses

The AF5 cell cultures used were the same as those described above for the long-term growth experiments. Genomic DNA was extracted from early, mid, and late-passage AF5 cultures, as well as from freshly dissected normal E13 rat fetal brain and tail tissues, using a Qiaex Genomic DNA Extraction kit (Qiagen Inc, Chatsworth, CA) following the manufacturers instructions. Oligonucleotides for polymerase chain reaction (PCR) were custom synthesized (Gibco BRL Life Technologies) to anneal to the intron sequences flanking exons 5, 6–7, and 8–9. The primer sequences were described previously (56). DNA (0.2–1 μg) was added to a reaction mixture containing 20 nM of each oligonucleotide primer and PCR Supermix (Gibco BRL Life Technologies) in a 100-μl volume and subjected to amplification in a Perkin–Elmer thermocycler for 30 cy-

cles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and elongation (72°C, 30 s). The amplification products were separated on 2% PE Express agarose gels (Perkin–Elmer, Foster City, CA) containing ethidium bromide. The DNA bands were visualized under UV light, quickly excised, then purified using kit reagents from Qiagen. DNA sequencing was performed in both the forward and reverse directions by the Johns Hopkins DNA Facility, using the PCR primers as the sequencing primers. The resulting sequences were compared for homology to published sequences by BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, MD)

p53 Activity

Activation of wild-type p53 was measured by a transient transfection assay as described previously (6, 10, 34). AF5 cells and primary fetal rat mesencephalic cells were subcultured into 12-well plates and grown to approximately 80% confluence. The PG13-Luc and MG15-Luc plasmids (kindly provided by B. Vogelstein, Johns Hopkins University) were used to detect p53 activation. The PC13 plasmid contains 13 copies of the p53 binding site upstream from the polyoma promoter and a luciferase reporter construct (12). The MG15-Luc negative control plasmid contains 15 copies of a mutated form of the p53 enhancer which does not bind wild-type p53. Briefly, transfections were carried out using DOTAP reagent according to the manufacturer's recommendations for transient transfection (Boehringer–Mannheim). Adriamycin (0.2 μg/ml, Sigma) was added to the cultures using three different treatment conditions: 24 h prior to transfection ("before"), immediately after transfection for 24 h ("after"), or both 24 h before transfection and again just after transfection ("both"). Luciferase was measured 24 h after transfection with the Luciferase Assay System (Promega). Each condition was assayed in triplicate wells.

Propagation of AF5 Cells in Serum-Free Media

AF5 cells were grown in serum-free media consisting of Neuralbasal media with 0.5 mM glutamine and either B27, G5, or N2 supplement (Gibco Life Technologies), in 25-cm^2 Biocoat culture flasks (Becton Dickinson, Bedford, MA) or multiwell plates that had been coated with laminin, collagen I, collagen IV, fibronectin, poly-D-lysine, or poly-L-ornathine/laminin, or no additional coating. In subsequent experiments, cells were plated directly into Costar culture flasks with Neuralbasal media containing glutamine and B27 supplement (NB–B27), or cells already growing in serum-containing media were switched to NB-B27 media. Adherent cultures were passaged by dissociation with trypsin/EDTA (Gibco Life Technologies). Spheres of cells suspended in the media were harvested by centrifugation of the culture media. For some experiments, spheres were transferred into flasks using serum-containing media with or without 1 μM dibutyryl cyclic AMP (dBcAMP; Sigma).

Comparison of Adherent AF5 Cells Growing Exponentially and Confluent

AF5 cells in serum-containing media were plated in 25-cm^2 flasks at 5×10^5 cells per flask. Over a 3-week period, one set of flasks was passaged every 2 days with a fresh change of media to maintain exponential growth, while sister cultures were allowed to become confluent without a change of media. Changes in morphology were observed and recorded, and immunocytochemistry, described earlier, was performed to characterize cell types. Each experiment in which properties of exponentially growing and confluent/differentiated cultures were compared was performed at the same time on sister cultures.

Proliferation of actively growing vs differentiated cultures was compared by 5-bromo-2′-deoxuridine incorporation (BrdU, 20 μM final concentration, PharMingen). The optimal time for BrdU incubation required for maximal detection of dividing cells was first established by a time course in actively growing cells. In experiments comparing actively growing vs differentiated cultures, BrdU was applied to the cultures for 19 h, in order to label the majority of cells in the actively growing condition. Cells were fixed for 30 min with 95% ethanol at −20°C, rehydrated and washed with culture media without serum (DMEM/F12), permeabilized with 2 N HCl for 10 min at room temperature, and the pH normalized with three or four washes of DMEM/F12. Nonspecific antigens were blocked with buffer containing 5% normal goat serum and 0.1% Nonidet P-40. Primary monoclonal antibody against BrdU (PharMingen) diluted 1:100 in blocking solution was applied overnight at 4°C. Following washes, Alexa 594 goat anti-mouse antibody was applied, the nuclei were counterstained with DAPI, and images were captured as described earlier. The mean percentage of BrdU-stained cells for each condition was calculated from counts of DAPI-stained and BrdU stained nuclei in five fields per flask.

15K cDNA Nylon Membrane Microarray

A cDNA microarray consisting of approximately 15,000 genes (Human Image Consortium cDNA clones (http://image.llnl.gov, Research Genetics) on nylon membranes was produced as described by Tanaka *et al.* (48), with modifications as described by Truckenmiller *et al.* (53).

TABLE 1

Growth Factors Secreted by AF5 Cells

	BDNF	PDGF	GDNF	TGFβ1	TGFβ2	bFGF	CNTF	EGF
AF5	31 ± 0.0	18130 ± 0.4	78 ± 0.0	1446 ± 0.1	142 ± 0.1	29 ± 0.0	ND	ND

Note. Data are the means ± SEM of triplicate samples, in pg/ml, which is approximately equivalent to pg/10^6 cells/day. ND, none detected.

Preparation of AF5 Cultures and RNA for Microarray Analysis

Gene expression by the AF5 cell line was compared under three conditions. Sister cultures of AF5 cells were plated in 75-cm^2 flasks, three flasks for each condition. The conditions were (a) cells maintained in exponential growth by thinning to 5×10^5 cells per flask every 2 days, (b) cells maintained in exponential growth with the addition of basic fibroblast growth factor (bFGF, 20 ng/ml, Gibco Life Technologies) to the media, and (c) cultures allowed to become confluent without thinning or changing the media. After 3 weeks, total cellular RNA was extracted directly in the flasks using RNAzol B (Tel-Test, Friendswood, TX), according to the manufacturer's suggestions, and the resulting RNA from the three flasks of each condition was pooled. The concentration and quality of the RNA was assessed by spectrophotometry and by agarose gel electrophoresis. RNA samples were stored at −80°C until used.

Microarray Procedure

RNA samples from AF5 cells were radiolabeled and hybridized to the 15K arrays essentially as described by Vawter *et al.* (57) with modifications as described by Truckenmiller *et al.* (53). The general procedure for probe preparation (radiolabeling of total RNA with [^{33}P]dCTP) can also be found http://www.grc.nia.nih.gov/branches/rrb/dna.htm. Briefly, for each sample, 5 μg total RNA was radiolabeled with [^{33}P]dCTP in a reverse-transcription reaction and purified using Bio-Rad 6 purification columns (Hercules, CA). The cDNA microarray membranes were prehybridized for 4 h at 50°C with rotation, and then approximately 5×10^6 cpm/ml of heat-denatured probe was added directly to the prehybridization solution. Hybridization was performed for 16–18 h at 50°C with rotation. After washes with 2× SSC, 0.1% SDS at room temperature, the membranes were exposed to phosphorimager screens for 7 days. The screens were then scanned in a Molecular Dynamics 860 PhosphorImager (Sunnyvale, CA) at 50 μm resolution. ImageQuant software (Molecular Dynamics, Sunnyvale, CA) was used to convert the hybridization signals on the image into raw intensity values, and the data thus generated was transferred into Microsoft Excel spreadsheets, predesigned to associate the Image Quant data format to the correct gene identities.

Microarray Data Analysis

Raw intensity data for each experiment were normalized by Z transformation (53, 57). Intensity data were first $\log_{10}$ compressed followed by the calculation of Z scores. Z scores were calculated by subtracting the $\log_{10}$ value of the average gene intensity from the $\log_{10}$ value of the raw intensity datum for each gene, and dividing that result by the $\log_{10}$ value of the standard deviation (SD) of all the measured intensities, according to the formula:

$$Z = (\log_{10}\text{ intensity} - \text{mean}\log_{10}\text{ intensity})/\log_{10}\text{ SD}$$

Gene expression differences between two conditions were calculated by finding the difference between the gene Z scores. Z ratios were then calculated for comparison between the different experimental conditions. To determine Z ratios, Z differences were divided by their appropriate conjugate standard deviation as in the formula:

$$Z\text{ ratio} = (Z_1 - Zn_2)/\text{SD}\ \Sigma\ (Zn_1 - Zn_2)$$

Z ratios are commonly used in multiple comparisons (similar to traditional fold-differences) without further reference to the standard deviations by which they were derived. GeneSpring software Version 3.6.2 (Silicon Genetics, Redwood City, CA) was used for data analysis and graphs.

RESULTS

Growth Factor Production

AF5 cells produced large amounts of PDGF (18130 ± 0.4 pg/ml), considerably lower amounts of TGFβ1 (1446 ± 0.1) and TGFβ2 (142 ± 0.1), and small but detectable amounts of GDNF (78 ± 0.0), BDNF (31 ± 0.0), and bFGF (29 ± 0.0), while CNTF and EGF were undetectable. Values are approximately equal to pg/10^6 cells/day (Table 1).

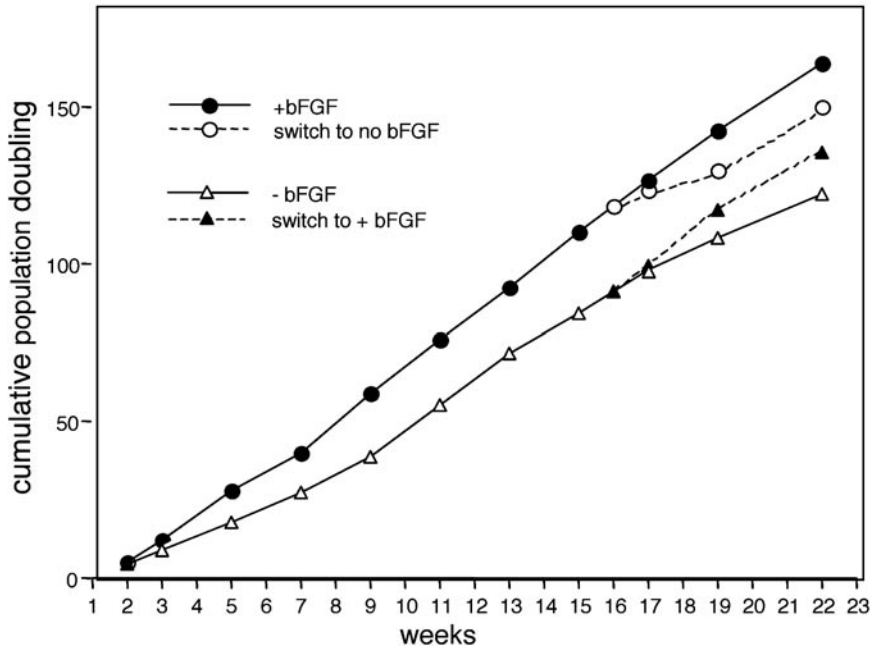

FIG. 1. Long-term growth of AF5 cells. Cells were grown continuously for 23 weeks, passaged each week to maintain exponential growth, either with or without bFGF in the media. At week 17, sister cultures from each condition were switched to the opposite condition. Cumulative population doublings (PD) were calculated at the indicated time points.

Long-Term Growth of Adherent AF5 Cultures

Figure 1 depicts the cumulative population doublings (PD) of early passage AF5 cells that were seeded at 2×10^5 cells in 25-cm^2 flasks, with or without bFGF, and passaged at that density each week for 23 weeks. Under these conditions the cultures never became confluent and the cells remained in log-phase growth. Growth curves over a 5-day period were obtained at the time points indicated, and PD were calculated as described under Methods. These data show that there were no signs of senescence, as would be indicated by a slowing of growth, for over 150 population doublings. At week 17, sister cultures from each condition (plus or minus bFGF) were switched to the opposite condition. Cells grew faster with bFGF than without, and the rates remained consistent until switched. Switched cells responded to the new condition by growing faster with bFGF or slower when bFGF was removed. Thus the cells remained responsive to bFGF after long-term culture, which also points to stability and preservation of plasticity.

Karyotype

Karyotype analyses were performed on the AF5 cells at early and late passage (over 50 PD) as well as on normal primary mesencephalic glial cultures from pooled rat fetuses, for comparison (Diagnostic Cytogenetics Inc). The ideograms presented in the primary source articles (8, 41) did not always agree as to chromosome identification and orientation in normal *Rattus norvegicus* cells, particularly for chromosome pairs 15 and above. For consistency, the ideogram presented by Satoh *et al.* (41) was used as the model, and the nomenclature and karyotypes refer to this ideogram.

In primary mesencephalic glial cultures, chromosomal abnormalities were seen in six of seven cells analyzed, including chromosomal deletions in two cells, altered chromosomes in two cells, and the presence of additional marker chromosomes or additional chromosomal material of unknown origin in three cells. The modal number of chromosomes was 42 (three cells) with 39, 41, 43, and 43 chromosomes present in the remaining four cells. Five of the seven cells were female (XX), and the remaining two were unclear. The modal number of chromosomal abnormalities was one, with the range being from zero to three.

Early passage AF5 cells were female (XX), with additional X chromosomes or partial X chromosomes in three of six cells. Most cells examined were tetraploid, and the modal number of chromosomal abnormalities was 8, with the range being 5 to 14. An abnormality common to all cells was the presence of additional material on chromosome 10 at band q33. The chromosomal abnormalities consisted mainly of deletions (37 of the 52 total abnormalities). There were no chromosomal fusions.

The late passage cells were essentially unchanged from the early passage cells. The cells were tetraploid, female (XX) with additional X chromosomes in most

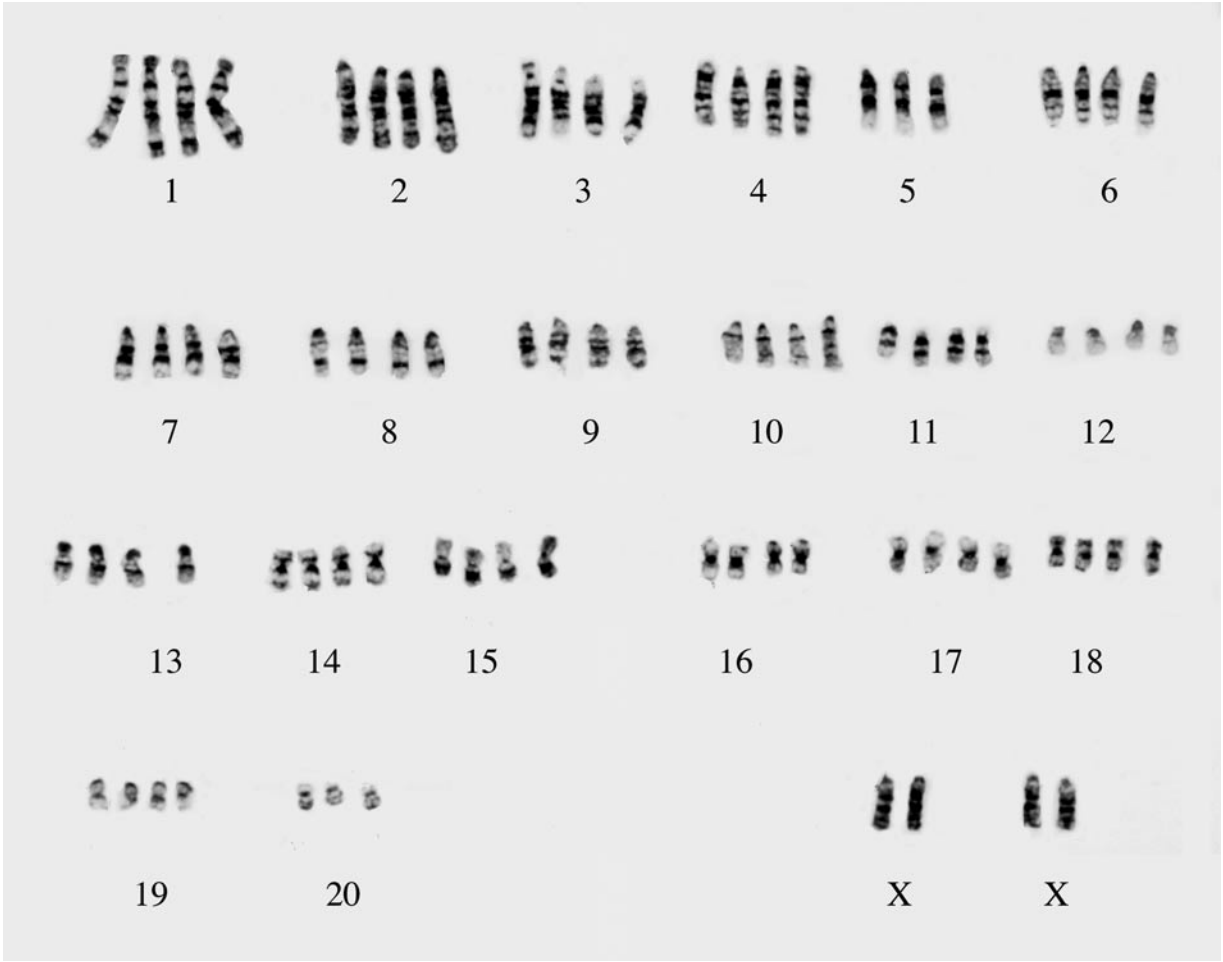

FIG. 2. Karyotype of a late passage AF5 cell.

cells analyzed (Fig. 2). The modal number of abnormalities was 7, with a range of 4 to 12. The same aberration common to the early cells, additional material on chromosome 10 at band q33, persisted in 10 of 11 cells analyzed. As with the early passage cells, abnormalities consisted mainly of deletions that were not common to all cells. Note that random loss of chromosomes can occur during preparation.

Telomerase Activity and Telomere Length in Long-Term AF5 Cultures

Using the same cultures as described in the legend of Fig. 1, telomerase activity and telomere length were assessed from early and late passage cultures of AF5 cells. Figure 3A shows that telomerase activity was relatively high and changed little between 3 weeks and 23 weeks in culture (approximately 120 population doublings, without bFGF). Telomere length assays also showed a single band at approximately 25 kb for all conditions, with no detectable changes in cells harvested during log-phase growth at weeks 2, 14, 20, and 23 in culture without bFGF, or weeks 3, 14, 20, and 23 with bFGF (Fig. 3B).

p53 Gene Sequence Analyses

Although T155g used to generate the AF5 cell line is lacking the region which binds and inactivates p53, culture conditions and/or the stress of immortalization in culture could result in genetic abnormalities or selection pressure which favored cells with a mutated p53 gene. We therefore sequenced the p53 gene in three regions corresponding to exons 5, 6–7, and 8–9, which were reported to be the most likely areas for mutations in rat tumor cells (56). In DNA extracted from early, mid, and late passage AF5 cultures, as well as freshly dissected normal fetal rat mesencephalic tissue, whole brain, and tail tissue, all sequences were 100% homologous to normal p53 gene or cDNA sequences (23). These results indicate that the p53 gene in AF5 cells was not mutated in the course of generating the cell line or after multiple passages in culture.

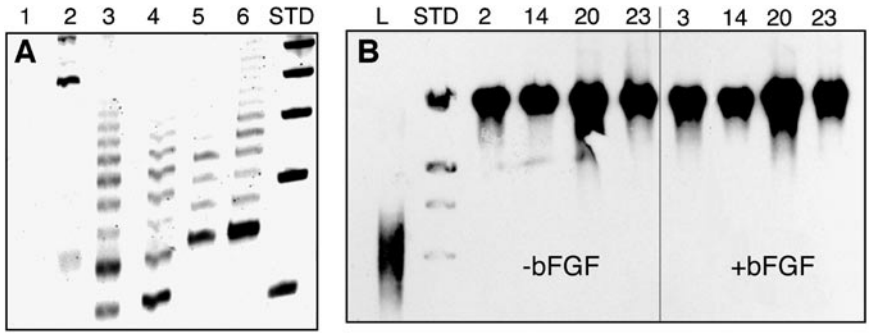

FIG. 3. Telomerase activity and telomere length of AF5 cells in long-term culture. Assays were performed on the same cells used for the long-term population doubling study shown in Fig. 1. (A) Telomerase activity measured by TRAP assay. Lane 1, PCR negative control; 2, heat-inactivated sample; 3, 3 weeks in culture; 4, 23 weeks in culture; 5, PCR-positive control; 6, 293 cells (positive control); STD 20 base pair size markers. (B) Telomere length assay. Lane L, low molecular weight control sample; STD, size markers of 23.1, 9.4, 6.6, and 4.4 kb, from top to bottom; remaining lanes, cells were grown continuously in culture for 2, 14, 20, and 23 weeks without bFGF or 3, 14, 20, and 23 weeks in culture with bFGF, as indicated.

p53 Functional Activity

A luciferase reporter assay was employed to measure p53 activation in response to adriamycin in the AF5 cell line as well as in normal primary fetal mesencephalic cultures. Cells were tested without adriamycin treatment and under three adriamycin treatment conditions: "before" was adriamycin treatment 24 h preceding transfection; "after" was adriamycin treatment immediately following transfection; "both" was adriamycin treatment both preceding and following transfection. In all cases luciferase activity was measured 24 h after transfection. (Fig. 4).

In both the AF5 cell line and primary fetal cells, overall significant increases in luciferase activity were observed in the cultures transfected with the PG13-Luc plasmid, as compared to the control MG15-Luc plasmid. In each cell type, one or more treatment conditions resulted in a significant increase, while the increase was not statistically significant under the control untreated condition. AF5 cells showed evidence for significant p53 activation with the "before" and "both" treatment conditions ($p < 0.05$); however, activation did not reach statistical significance ($p = 0.064$) for the "after" condition (Fig. 4A). For mesencephalic glia, significant adriamycin-induced activation was seen only for the "both" treatment condition ($p = 0.032$) (Fig. 4B).

AF5 Growth Conditions

Because the AF5 cell line appeared to have some characteristics of both astrocytes and neurons (52), we assessed growth and phenotype in conditions which favor the growth of neurons (B27 supplement in serum-free Neuralbasal media), neurons and astrocytes (N2 supplement), or astrocytes (G5 supplement), as well as the effects of different substrates on cell adhesion. In serum-free conditions, the cells flourished in Neuralbasal media containing B27 supplement (NB-27) and attached most readily to surfaces with collagen or laminin coating, albeit weakly as compared with cells in serum-containing media. The cells did not survive passaging in the other supplements. The soma of cells in NB-27 media that were attached to collagen or laminin coating were rounded and compact, with a

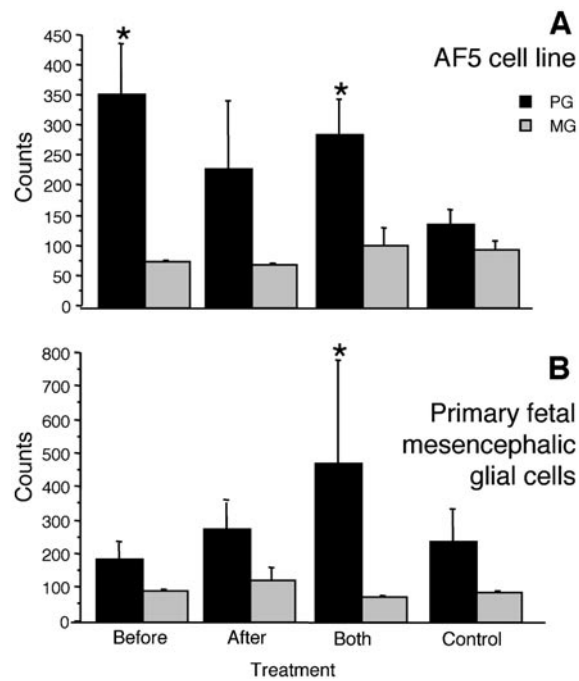

FIG. 4. Adriamycin-induced wild-type p53 activity. AF5 cells (A) and primary mesencephalic glial cells (B) were tested for p53 activity without adriamycin and under three adriamycin treatment conditions: "Before" (adriamycin treatment 24 h preceding transfection for 24 h), "After" (treatment immediately following transfection for 24 h), "Both" (treatment both preceding and following transfection), and "Control" (no adriamycin). In all cases luciferase activity was measured 24 h after transfection using the PG13-Luc reporter plasmid (PG), and the control MG15-Luc plasmid (MG). Data are means ± SEM. *Significant activation ($p < 0.05$).

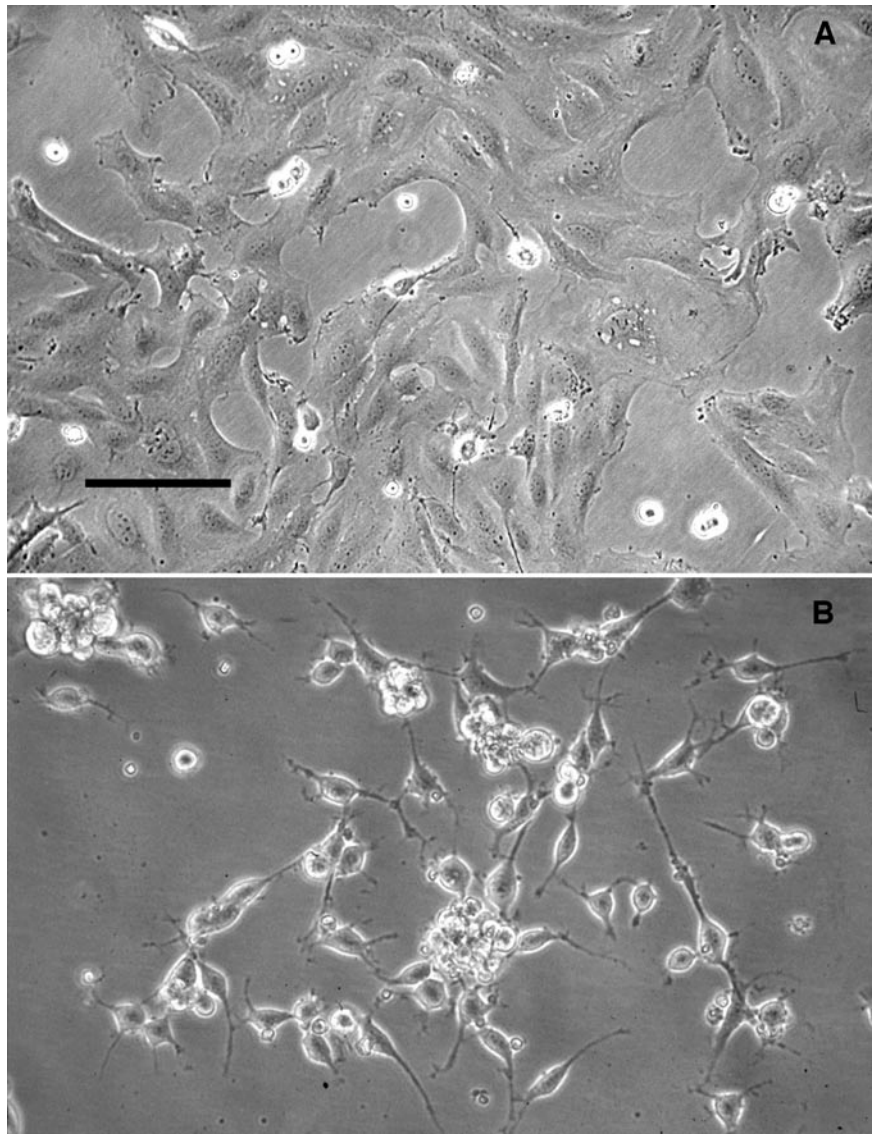

FIG. 5. Medium effects on morphology of AF5 adherent cultures. (A) Live culture, phase contrast, in exponential growth phase, in 10% FCS-containing media; (B) Cells in NB-27 serum-free medium on collagen substrate.

phase-bright appearance, and most cells were unipolar or multipolar, rather than having the flat fibroblast-like shape of AF5 cells in serum-containing media (Figs. 5A and 5B). Cultures in NB-27 media in flasks without collagen or laminin coating proliferated predominantly as spherical aggregates in suspension.

Propagation as Neurospheres

AF5 cells that were growing as adherent cultures in serum-containing media (10% FCS) were either switched to serum-free NB-27 media in the same flasks or passaged into new untreated flasks, after washing to remove serum, using NB-27 media. In the first case, the adherent cells began to round up within 1 or 2 days, and after about 1 week numerous cells were detached and suspended in the culture media. In the second case, few cells attached to the flask substrate while most remained suspended. In both situations, the suspended cells continued to divide and formed multicell spheres. Spheres appeared to approximately double in size in 24–36 h. These spheres could be propagated in this form, either in conditioned media or fresh NB-27 media. Spheres dissociated into smaller spheres or single cells could also be passaged in NB-27 media and continued to proliferate as floating spherical aggregates. Figure 6A depicts AF5 cells growing as spheres. Spheres of AF5 cells placed into 10% serum-containing media began to adhere to untreated culture flasks within 1 day and continued to proliferate as attached cells (Fig. 6B). Within 2–5 days all cells were adherent

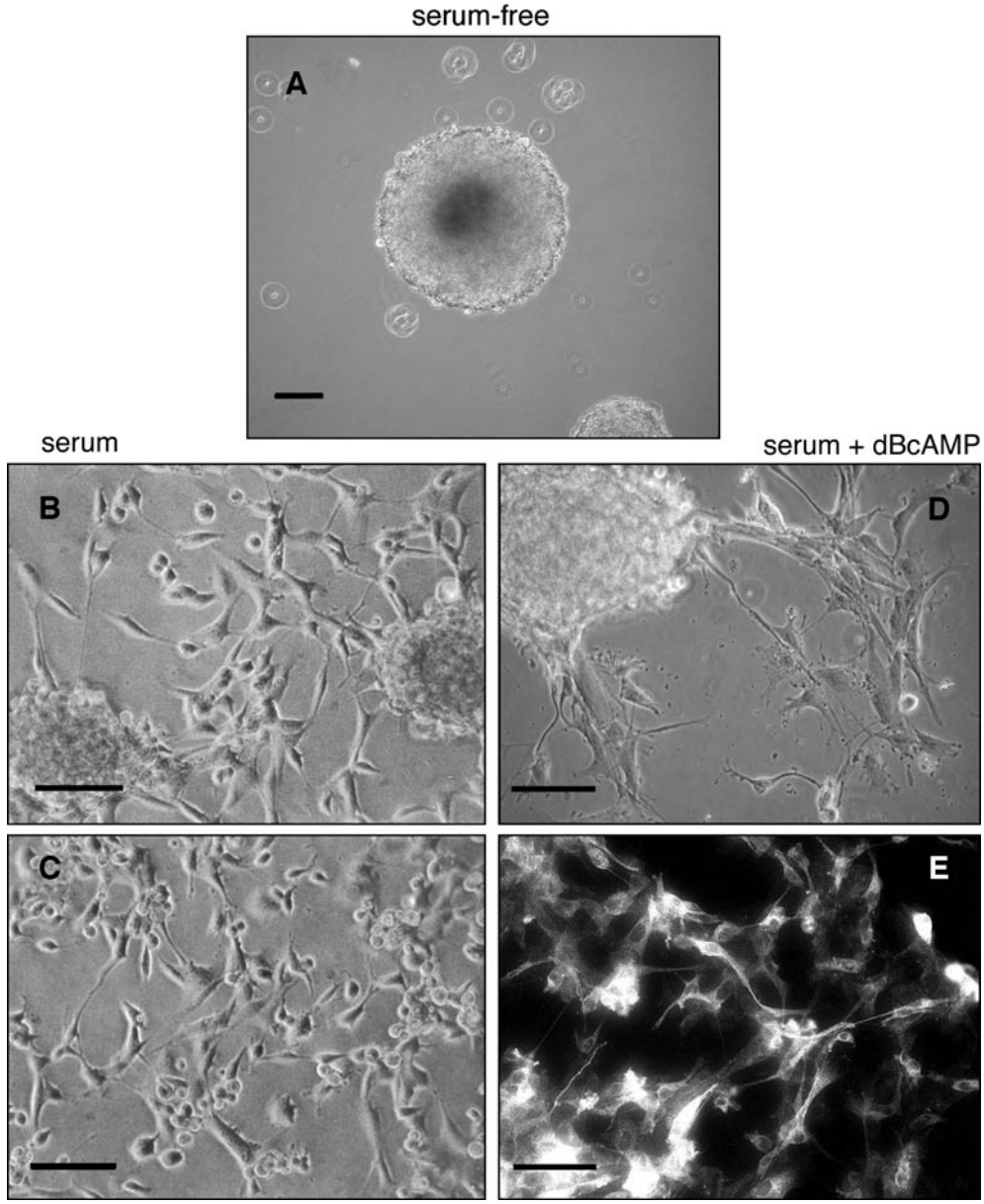

FIG. 6. AF5 spheres and differentiation. (A) Example of an AF5 sphere-aggregate in NB-27 serum-free medium; (B) Spheres adhering to plastic culture substrate 1 day after addition of 10% FCS; (C) Same culture as B, day 5; (D) Spheres with serum plus dBcAMP, day 1; (E) Same culture as D, immunostained with βIII-tubulin marker for neurons, day 3. Scale bars, 25 μm.

(Fig. 6C). This process was reversible; the cells detached when switched to NB-27 and reattached when serum was added, and continued to proliferate in either condition.

AF5 spheres switched to media containing 10% FCS with or without 1 μM dBcAMP readily settled and attached onto the flasks. Within 1 day, cells grew out from the cell mass and adhered to the flask. Cultures with dBcAMP showed extended processes, a morphological change indicative of differentiation (Fig. 6D). These cells immunostained for the neuron-specific marker βIII-tubulin (Fig. 6E)

Adherent Cultures Spontaneously Differentiate

The AF5 cell line spontaneously differentiated into cells with different morphologies when allowed to become confluent and undisturbed for 2–4 weeks in culture flasks with 10% serum-containing media. To characterize the changes associated with differentiation, sister cultures were either passaged every 2–3 days to maintain subconfluent exponential growth or allowed to become confluent and differentiate over 3 weeks without changing the medium. In the confluent cultures, most cells ceased dividing, and gradually devel-

oped complex patterns and multiple morphologic forms consisting of a base layer of broad flat cells with an overlay of cells having long processes (compare Figs. 7A1 and 7B1). The cells could remain in this quiescent differentiated state for several weeks. Cells that were differentiated in this manner could be harvested and passaged; however, they appeared to retain a heterogeneous morphology. BrdU incorporation (19 h) was observed in 79.9 ± 2.7% (mean ± SEM; total 189 cells examined) of freshly passaged AF5 cells, but was detected in only 1.1 ± 0.19% (total 3294 cells) of the cells in 2-week differentiated cultures (Figs. 7A2 and 7B2).

The cultures developed from immature to mature neuronal phenotypes upon differentiation. Double-immunostaining for astrocyte-specific (GFAP) and neuron-specific (βIII-tubulin) markers showed that the cells maintained in exponential growth immunostained predominantly for GFAP, while a βIII-tubulin-positive phenotype emerged in the differentiated cultures (Figs. 7A3 and 7B3). The GFAP and βIII-tubulin staining in the differentiated cultures appeared to be in distinctly different subsets of cells with no detectable evidence of coexpression of these markers in the same cells (this distinction was readily observed by viewing the cells in different focal planes). Cultures stained for βIII-tubulin alone showed a pattern similar to the double-stained cultures: few or no positive cells were detected in the subconfluent exponentially growing cultures, whereas large numbers of cells (although not all) were positive in the differentiated cultures (Figs. 7A4 and 7B4). The density and irregular shapes of the differentiated cells did not permit accurate counts of βIII-tubulin-positive cells, but the difference between the growing vs differentiated cultures was obvious in all cultures examined. Approximately 1% of the cells in confluent cultures were strongly immunopositive for tyrosine hydroxylase (not shown).

Nestin is an intermediate filament protein associated primarily with immature or developing cells. Seventy-six percent (± 5%; three flasks examined) of exponentially growing AF5 cells immunostained for nestin, while only two small patches (two or three cells per patch, or about six cells total) of nestin-positive cells were found throughout three entire flasks of 2-week differentiated cells containing several million cells (Figs. 7A5 and 7B5).

Microarray Gene Expression Patterns in AF5 Cells

A 15,000 (15K) gene microarray analysis was performed on AF5 cells grown in three different conditions: (a) subconfluent exponential growth in serum-containing media without bFGF ("normal" growth), (b) exponential growth in serum-containing media with 20 ng/ml bFGF, and (c) differentiated confluent cultures (no FGF and no media change for 3 weeks). Raw intensity values were normalized into *Z* scores for each array, and *Z* ratios, which serve a similar role as traditional fold-differences, were calculated for comparing gene expression differences. Genes exhibiting a *Z* ratio = 0 are considered to be neither increased nor decreased. The complete data set can be accessed (http://www.grc.nia.nih.gov/branches/rrb/dna/dnapubs.htm).

Genes Expressed in AF5 Cells in All Three Conditions

Table 2 lists a selection of genes that showed high expression (*Z* score ≥ 0.8) in all three conditions, grouped according to CNS cell type markers, neurotransmitter receptors, growth factor receptors, and adhesion/recognition molecules. This list indicates some genes that are characteristic of specific cell types and cellular functions, and provides an overview of the gene expression profile of these cells.

Comparisons of Gene Expression Levels

Figure 8 shows scatter plots of *Z* score intensity values illustrating patterns of increased or decreased gene expression. Comparisons include exponential growth conditions with and without bFGF (Fig. 8A), and exponential growth condition without bFGF to the differentiated condition (Fig. 8B). The identity of any point on the plots can be obtained via the GeneSpring program. Many points on both plots with very high *Z* scores were expressed sequence tags (ESTs), which were sequences of unknown identity at the time of this writing. Note that the distribution of points along the linear regression axes is a good indicator of the reliability of the data.

As described earlier, *Z* ratios were calculated to compare gene expression changes in the different growth conditions. In each case, the data for the normal exponentially growing cells was used as the "control." Thus positive *Z* ratios in the differentiated condition represent increased expression in the differentiated/confluent condition as compared with the normal "control" condition, and negative *Z* ratios indicate decreased expression. Similarly, the bFGF-stimulated condition was compared to the normal growth condition, and so positive *Z* ratios indicate increased expression with bFGF, whereas negative *Z* ratios indicate decreased expression. *Z* ratios measure expression of an individual gene as compared to the distribution of expression of the other genes in the array and are thus not directly related to numerical ratios. Often *Z* ratios will represent greater differences than typical fold-differences due to log-compression of the data. Table 3 contains lists of named genes (ESTs and unknown genes excluded), filtered by *Z* ratios ≥ 2.0, that were increased or decreased in the differentiated condition or in the +bFGF condition, and grouped into general functional categories of the gene products. The total number of named genes from which these lists were compiled was

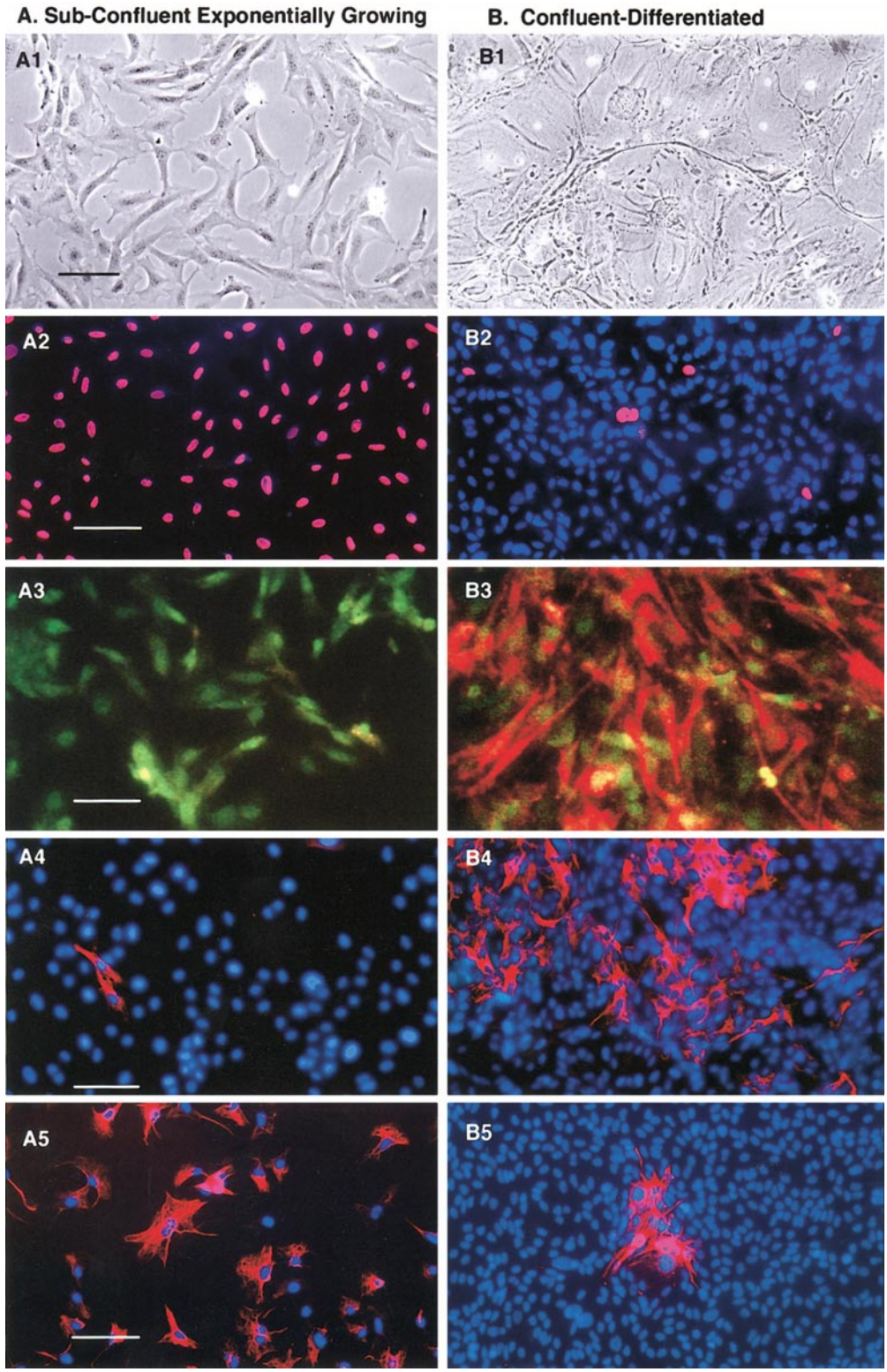

FIG. 7. Comparison of AF5 cells during exponential growth and when differentiated under confluent conditions. Column A images are exponentially growing AF5 cultures, and column B images are the differentiated sister cultures to those in column A. (A1 and B1) Phase contrast images of live cultures; (A2 and B2) fluorescent detection of BrdU incorporation of proliferating cells (red) with DAPI nuclear counterstain of all cells (blue) (BrdU-positive nuclei appear magenta due to overlap with the blue DAPI stain); (A3 and B3) cells double immunostained for GFAP (green) and βIII-tubulin (red); (A4 and B4) βIII-tubulin (red) with DAPI nuclear counterstain (blue); (A5 and B5) nestin (red) with DAPI counterstain (blue). The small cluster of nestin-stained cells in B5 was one of three such clusters found throughout three flasks containing several million cells. Scale bar is 25 μm in A1, B1, A3, B3, and 35 μm in A2, B2, A4, B4, A5, B5.

TABLE 2

Selected Neural-Relevant Genes Expressed in AF5 Cells (Z score > 0.8)

Locus Link	Clone ID	Accession No	Gene Name (symbol)	Z score		
				Differentiated	+bFGF	−bFGF
Genes representative of cell types						
4741	743229	AA400329	Neurofilament 3 (NEF3)	1.51	1.10	1.16
2670	382693	AA069414	Glial fibrillary acidic protein (GFAP)	0.86	0.98	1.75
8514	48631	H14383	Potassium voltage-gated channel, shaker-related subfamily, beta member 2 (KCNAB2)	1.91	1.50	1.88
6811	787857	AA452374	Syntaxin 5A (STX5A)	2.22	1.96	2.41
6622	812276	AA455067	Synuclein, alpha (non A4 component of amyloid precursor) (SNCA)	1.79	1.39	2.88
Receptors						
154	241489	H90431	Adrenergic, beta-2-receptor, surface (ADRB2)	1.23	1.71	1.33
1268	26295	R20626	Cannabinoid receptor 1 (brain) (CNR1)	0.85	1.08	0.88
Growth factor-related						
1382	897770	AA598508	Cellular retinoic acid-binding protein 2 (CRABP2)	5.00	5.14	4.83
3588	842860	AA486393	Interleukin 10 receptor, beta (IL10RB)	3.42	3.53	3.27
6424	841282	AA486838	Secreted frizzled-related protein 4 (SFRP4)	2.28	2.28	3.18
5157	810010	AA455210	Platelet-derived growth factor receptor-like (PDGFRL)	2.04	2.21	2.41
3482	79712	T62547	Insulin-like growth factor 2 receptor (IGF2R)	2.34	2.31	1.89
5159	40643	R56211	Platelet-derived growth factor receptor, beta polypeptide (PDGFRB)	4.81	4.48	4.23
7048	841149	AA487034	Transforming growth factor, beta receptor II (70-80kD) (TGFBR2)	1.77	1.71	1.07
Adhesion/recognition molecules						
6900	28510	R40446	Contactin 2 (axonal) (CNTN2)	1.44	1.63	1.31
1001	773301	AA425556	Cadherin3, P-cadherin (CDH3)	3.09	3.14	2.65
1003	69672	T53626	Cadherin5, VE-cadherin (vascular epithelium) (CDH5)	1.09	1.61	2.13
1499	774754	AA442092	Catenin (cadherin-associated protein), beta 1 (88kD) (CTNNB1)	1.15	1.11	1.95
2051	1031552	AA609284	Ephrin-B6 (EPHB6)	1.20	0.93	0.99
2049	811088	AA485795	Ephrin-B3 (EPHB3)	1.72	2.12	1.80
6402	149910	H00756	Selectin L (lymphocyte adhesion molecule 1) (SELL)	1.15	0.92	1.20

3630 genes. Table 3A lists the genes with increased expression in the differentiated/confluent state as compared to normal growing cells; Table 3B shows genes with decreased expression in the differentiated as compared to the growing state. Similarly, Table 3C shows genes with increased expression in the bFGF-stimulated condition as compared to normal growing cells without bFGF, and Table 3D shows genes with decreased expression in the bFGF as compared to no FGF condition. These lists represent the genes that showed very large changes, and may not include genes that were changed but to a lesser degree. The complete gene expression data set is available at (http://www.grc.nia.nih.gov/branches/rrb/dna/dnapubs.htm).

DISCUSSION

This report describes two aspects of the AF5 cell line: (i) long-term growth properties related to immortalization and *in vitro* stability, and (ii) *in vitro* plasticity, including propagation as progenitor-like cells and characteristics of differentiation.

Continuous maintenance of cells in culture may select for subpopulations of cells with faster growth rates due to genetic mutations or abnormalities, and such faster growing cells ultimately overtake the original cells. Moreover, some immortalization strategies which interfere with p53 function may promote genetic instability. Early passage AF5 cells maintained a stable

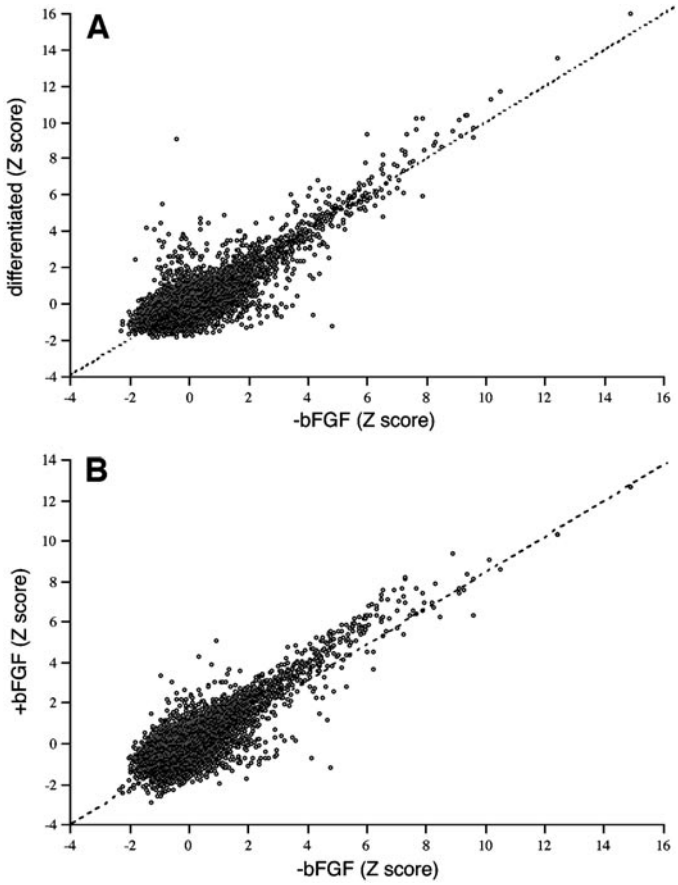

FIG. 8. Microarray 15K gene expression scatter plots. The relative intensity values (*Z* scores) for all genes were plotted for the -bFGF growth condition against intensity values for the +bFGF condition (A) and the differentiated condition (B).

growth rate over 23 weeks in continuous culture, or more than 150 population doublings, and remained responsive to bFGF. These observations indicate that genetic alterations that would effect growth rate did not occur over long-term culture.

Oncoproteins that interfere with p53 activity may contribute to genetic instability by preventing apoptosis of aberrant or mutated cells. Full-length SV40 large T protein (LT), including temperature-sensitive variants, is comprised of domains that bind and inactivate cellular proteins including p53. The AF5 cell line, established using a fragment of LT, which lacks the p53-binding domain (T155g), was assumed to have some protection from developing certain aberrancies by maintaining intact p53 mechanisms. It is possible, however, that during the process of immortalization or during long-term culture the p53 gene could have undergone mutations resulting in cells with a growth advantage. However, AF5 cells had no mutations in the p53 gene, and adriamycin-induced p53 activity was also normal. These results support the supposition that p53 mutation did not play a role in immortalization of these cells, nor was it a factor in the continued growth of these cells in culture. Moreover, while the karyotype of AF5 cells was not completely normal, the pattern did not change significantly over long-term culture, indicating genetic stability of these cells.

AF5 cells expressed telomerase in early passages and throughout long-term culture, and telomere length remained unchanged, which is consistent with normal rodent neural precursor cells (35). Whereas *in vitro* senescence of human cells is believed to be related to telomere shortening, senescence of rodent cells may be independent of telomere length, and instead caused by stress or progressive injury that occurs in suboptimal culture conditions (42).

There are, however, senescence-like phenomena which may appear when rat cell lines are extensively passaged. Normal rat tissues have telomeres which vary from 20–100 kb in length (31). Telomere length is expected to vary substantially with prolonged passaging of cell lines. There have, however, been very few direct studies of telomere maintenance in rat cell lines. Golubovskaya and coworkers (18) examined telomere maintenance in WB-F344, a cell line derived from normal rat liver by passaging without the use of an oncogene (55). Cell lines derived in this manner, by passaging, generally exhibit a loss of p53 and/or $p19^{ARF}$ function (26, 63) in contrast to AF5 which exhibits intact p53 and normal p53 function (normal p53 function is indicative of normal $p19^{ARF}$ function as well). With extended growth for very long periods of time *in vitro*, WB-F344 cells showed highly variable telomere lengths and activity. Telomerase activity disappeared after passage 11, but reemerged at passages 104–221. Telomere length was also variable, between 15–100 kb at passage 8, with erosion of telomeres and changing banding patterns between passages 31 and 234. In addition, marked chromosal instability was seen, especially at intermediate passage numbers (which correspond to the long-term culture durations used in the present report). Although it is risky to draw conclusions from a comparison of only two cell lines, it seems that the AF5 cell line shows a considerably higher degree of genetic stability than one other rat cell line that has been similary studied, WB-F344, which was produced by alternative methods.

Recent reports (32, 49) propose that normal primary rodent cells can proliferate indefinitely in culture in the absence of oncogene expression or a mutational event, provided the proper culture conditions are found. However, an oncogene may yet be required to facilitate this process for some cell types that have a limited ability to divide spontaneously (e.g., 27). We are confident that T155g-established cell lines did not arise spontaneously, since only cells expressing the transgene have ever survived G418 selection and long-term culture (unpublished observations in establishing numerous CNS cell lines). A “mild” oncoprotein such as

TABLE 3

Category	*Z* ratio	Locus Link	Clone ID	Accession	Gene name
A. Differentiated Increased. Genes with increased expression in differentiated state (confluent)					
Signaling, neurotransmission and synaptic function, second messenger systems	10.02	1428	42373	R67147	Crystallin Mu
	4.59	2903	46054	H08933	*N*-methyl-D-aspartate receptor modulatory subunit 2A (hNR2A)
	4.31	5531	772455	AA405562	Protein phosphatase 4 catalytic subunit
	4.11	928	727251	AA412053	CD9 antigen
	4.04	47	502622	AA136054	ATP-citrate lyase
	3.52	3628	180803	H52141	Inositol polyphosphate-1-phosphatase
	2.76	6457	179283	H50251	SH3 domain protein 2C
	2.48	2560	35185	R24969	Gamma-aminobutyric acid (GABA) A receptor, beta 1
	2.34	5026	486678	AA044267	Purinergic receptor P2X, ligand-gated inon channel, 5
	2.34	5026	486678	AA044267	Purinergic receptor P2X, ligand-gated inon channel, 5
Structural, filaments, cytoskeleton	3.31	3853	592111	AA150532	Keratin, type II cytoskeletal 6D
	3.03	6699	813614	AA447835	Small proline-rich protein 1B (cornifin)
Cell cycle control, DNA function, chromosomes, apoptosis	5.95	1105	262996	H99736	Chromodomain-helicase-DNA-binding protein (CHD1)
	3.57	7163	814306	AA459318	Tumor protein D52 (N8 protein)
	3.52	29966	767994	AA418918	Cell cycle nuclear autoantigen GS2NA
	2.73	834	120106	T95052	Caspase 1, apoptosis-related cytein protease
	2.43	8887	898109	AA598483	Tax1-binding protein (TAX1BP1)
	2.04	597	814478	AA459263	Bcl-2 related (Bfl-1)
	2.02	4170	843287	AA488674	Myeloid cell leukemia sequence 1 (BCL2-related)
Adhesion, extracellular matrix, membrane glycoproteins	2.49	1272	24884	R38995	Contactin 1
	2.14	9308	530185	AA111969	CD83
Growth/trophic factors and receptors, development	5.58	2489	341310	W58032	Human frezzled (fre)
	4.26	3569	310406	N98591	Interleukin 6
	2.38	1908	66532	T67005	Endothelin 3
	2.36	10920	824382	AA489699	COP9 homolog (HCOP9)
	2.03	54741	194182	H51066	Leptin receptor gene-related protein
Transcription, translation, RNA processing	6.74	5936	811911	AA456271	RNA binding motif protein 4
	4.59	7175	712641	AA280514	Translocated promotor region
	3.56	7110	684940	AA252318	tata element modulatory factor
	3.13	1994	345208	W72322	HuR RNA binding protein (HuR)
	2.90	6135	869450	AA680244	Ribosomal protein L11
	2.32	5463	289447	N63968	POU homeobox protein
	2.22	2958	73381	T55801	Transcription initiation factor IIA gamma chain
Protein processing, trafficing	3.68	3300	810053	AA465017	DNAJ protein Homolog HSJ1
	2.37	7428	234856	H73054	Von Hippel-Lindau syndrome (G7)
	2.13	1519	229901	H70866	Cathepsin O precursor
Metabolism, mitochondrial	3.60	1350	884480	AA629719	Cytochrome c oxidase VIIc subunit
	3.35	2949	277507	N56898	Glutathione *S*-transferase M5
	3.31	5350	773771	AA427940	Phospholamban
	2.65	128	813711	AA453859	Alcohol dehydrogenase 5 chi subunit (class III)
	2.50	4725	562409	AA214053	NADH-ubiquinone oxidoreductase 15-kDa Psubunit
	2.19	2876	810999	AA485362	Glutathione peroxidase1
	2.11	23456	193990	R83875	ATP-binding cassette subfamily B
Immune-related	2.78	3507	214441	H73590	Immunoglobulin mu C

TABLE 3—*Continued*

Category	*Z* ratio	Locus Link	Clone ID	Accession	Gene name
B. Differentiated Decreased. Genes with decreased expression in differentiated state (confluent)					
Signaling, neurotransmission and synaptic function, second messenger systems	−2.21	2059	148028	H13623	Epidermal growth factor receptor kinase substrate(Eps8)
	−2.02	51495	866694	AA679177	Butyrate-induced transcript 1
	−3.13	5609	223128	H85962	Mitogen activated protein kinase 7
	−1.56	23049	261580	H98694	PI 3 kinase related kinase SMG-1
	−2.07	10981	472186	AA057378	Ras-related protein (RAB32)
	−2.84	4306	784296	AA447079	Mineralocorticoid receptor (MR)
	−2.87	10244	249606	H84815	Rab9 effector p40
	−2.31	10728	884822	AA669341	Unactive progesterone receptor (p23)
	−2.24	1186	262251	H99364	Chloride channel protein 7 (CLCN7)
Structural, filaments, cytoskeleton	−2.26	8655	853938	AA644679	Dynein, cytoplasmic, light chain
	−2.81	4703	611586	AA176957	Nebulin
Cell cycle control, DNA function, chromosomes, apoptosis	−2.48	5728	322160	W37864	Phosphatase and tensin homolog
	−3.23	1874	786048	AA448641	E2F transcription factor 4, p107/p130-binding
	−2.94	9537	48285	H12189	P53 induced protein
	−2.15	699	781047	AA446462	Putative mitotic checkpoint protein kinase(HsBUB1)
Adhesion, extracellular matrix membrane glycoproteins	−2.45	1308	252259	H87536	Collagen type XVII, alpha 1
	−2.06	4017	882506	AA676458	Lysyl oxidase-like 2
	−2.74	3679	377671	AA055979	Integrin, alpha 7B
Growth/trophic factors and receptors, development	−2.44	2247	23073	R38539	Fibroblast growth factor 2 (basic)
Transcription, translation, RNA processing	−2.10	9987	897823	AA598578	Heterogeneous nuclear ribonucleoprotein D-like
	−2.29	10262	432564	AA699361	Spliceosomal protein (SAP49)
	−2.00	8565	841070	AA486761	Tyrosyl-tRNA synthetase
	−2.22	23650	377275	AA055486	Ataxia-telangiectasia group D-associated protein
Protein processing, trafficing	−2.16	3998	774420	AA446103	ERGIC-53 protein precursor
Metabolism/mitochondrial	−2.12	11332	471799	AA035455	Acyl-CoA thioester hydrolase
	−2.36	1629	195753	R89083	Dihydrolipoamide branched chain transacylase
	−2.28	3034	416803	W86776	Histidine ammonia-lyase
	−2.05	1337	840894	AA482243	Cytochrome C oxidase subunit Via
	−2.99	10449	45376	H07926	Mitochondrial 3-oxoacyl-CoA thiolase
	−3.23	23530	51826	H22944	NAD(P) transhydrogenase
	−2.10	7355	179753	H51549	UDP-galactose translocator
Immune-related	−4.47	3105	853906	AA644657	MHC class I protein HLA-A (HLA-A28, -B40, -Cw3)
C. bFGF Increased. Genes with increased expression during bFGF treatment.					
Signaling, neurotransmission and synaptic function, second messenger systems	3.10	10493	856902	AA669603	Membrane protein of cholinergic synaptic vessicles
	3.07	1819	813158	AA456688	Developmentally regulated GTP-binding protein(DRG2)
	2.40	2566	28218	R40790	Gamma-aminobutyric acid (GABA) A receptor, gamma 2
	2.09	5504	769657	AA428749	Protein phosphatase 1, regulatory (inhibitor) subunit 2
	2.03	393	212640	H69620	Rho-GTP-ase activating protein 4 (p115)
Structural, filaments, cytoskeleton	2.54	1783	811870	AA454959	Dynein light intermediate chain 2
	2.52	1411	839094	AA487614	Beta crystallin A3 (CYRBA3/A1)
Cell cycle control, DNA function, chromosomes, apoptosis	3.31	1642	950680	AA608557	Damage-specific DNA binding protein 1(127kD)
	2.21	3005	205445	H57830	H1 histone family, member 0
	2.02	3936	344589	W73144	Lymphocyte cytosolic protein1(L-plastin)

TABLE 3—*Continued*

Category	Z ratio	Locus Link	Clone ID	Accession	Gene name
Adhesion, extracellular matrix membrane glycoproteins	3.49	4185	184240	H43854	A disintegrin and metalloproteinase domain 11
	2.65	5167	82991	T70503	Ectonucleotide pyrophosphatase/ phosphodiesterase 1
	2.27	7087	180864	R87840	Intercellular adhesion molecule 5, telencephalin
	2.25	4974	51373	H24006	Oligodendrocyte myelin glycoprotein precursor
	2.11	8189	346552	W74377	Symplekin
	2.10	1833	503579	AA131238	Dermatan sulfate proteoglycan 3(DSPG3)
Growth/trophic factors and receptors, development	2.65	2173	345626	W72051	Fatty acid binding protein, brain
	2.37	1808	841620	AA487674	Dihydropyrimidinase related protein-2
	2.36	9255	182977	H43129	Small inducible cytokine subfamily E, member 1
	2.28	3600	289606	N59270	Interleukin15
	2.28	1435	73527	T55558	Colony-stimulatingfactor1 (M-CSF)
	2.17	2263	809464	AA443093	Fibroblast growth factor receptor 2
Transcription, translation, RNA processing	2.40	2962	711961	AA282092	General transcription factor IIF, polypeptide 1(74kD subunit)
Protein processing, trafficing	3.23	2289	416833	W86653	FK506-binding protein 5
	2.36	9040	785847	AA449119	Ubiquitin-conjugating enzyme E2M
	2.01	10558	843234	AA488447	Serine palmitoyl transferase I
Metabolism/mitochondrial	4.40	1571	179403	H50500	Cytochrome P450, subfamily IIE
	2.81	212	214443	H73591	Cytochrome B5
	2.21	501	563673	AA101299	Aldehyde dehydrogenase 7 family, member A1
	2.12	439	825677	AA504809	Arsenical pump-driving ATPase
	2.01	2167	307660	N92901	Fatty acid binding protein4, adipocyte
Immune-related	1.50	10493	853906	AA644657	MHC class I protein HLA-A (HLA-A28, -B40, -Cw3)
D. bFGF Decreased. Genes with decreased expression during bFGF treatment					
Signaling, neurotransmission and synaptic function, second messenger systems	−3.44	2794	257523	N30302	GTP-Binding protein HSR1
	−2.17	5876	812155	AA456028	Geranylgeranyl transferase type II beta-subunit
Structural, filaments, cytoskeleton	−2.30	2317	840818	AA486238	Filamin B, beta (actin-binding protein-278)
	−2.07	6709	76362	T60235	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
Cell cycle control, DNA function, chromosomes, apoptosis	−2.32	5921	815507	AA457027	GTPase-activating protein ras p21 (RASA)
	−2.19	4686	703739	AA278749	Nuclear cap binding protein, 80 kDa
Protein processing, trafficing	−7.3	10961	450307	AA682851	ERp31protein
	−2.34	826	882548	AA676484	Calpain, small polypeptide
	−2.32	5481	884500	AA629987	Cyclophilin 40
	−2.14	831	730002	AA416952	Calpastatin
Growth/trophic factors and receptors, development	−2.32	3428	824602	AA491191	Human interferon-gamma induced protein (IFI 16)
	−2.14	2252	365515	AA009609	Fibroblast growth factor 7 (keratinocyte growth factor)
	−2.09	92	450464	AA682819	Activin A receptor, type II precursor
Transcription, translation, RNA procesing	−2.12	7738	814014	AA455712	Zinc finger protein (ZNF184)
	−2.03	None	549101	AA083577	Ribosomal protein L19
Metabolism, mitochondrial	−3.15	None	151595	H03954	Glucose transporter pseudogene
	−2.05	2878	855523	AA664180	Glutathione peroxidase 3 (plasma) (GSHPX-P)

T155g may therefore be useful for immortalizing cells that cannot readily be induced to exhibit sustained growth *in vitro*. Moreover, identifying culture conditions appropriate for unlimited growth of all the various cell types that might be employed may not be feasible.

The AF5 cell line, when first established, was shown to express markers characteristic of both astrocytes and neurons in culture. In the present study, the AF5 cells similar to neural precursor cells, were shown to survive and propagate as spherical aggregates in suspension, or "neurospheres", in serum-free media with a supplement formulated for neurons (NB-27 media). Also, as described for neural progenitor cells, spheres grown in NB-27 medium attached to culture surfaces coated with collagen or laminin, or with addition of FCS to the media the cells attached without surface coatings. AF5 spheres exhibited a rapid differentiation response, extending processes and expressing the neuron-specific marker βIII-tubulin, with addition of serum and dBcAMP to the media. The addition of dBcAMP to neurosphere cultures freshly plated in serum-containing media did appear to induce or accelerate differentiation before the cells reached confluence; however, dBcAMP treatment was not required for differentiation.

Many studies on differentiation of CNS cells involve the use of tumor-derived or hybrid cell lines that are induced to differentiate with various agents such as retinoic acid, nerve growth factor, dBcAMP, glutamate, and others (e.g., 3, 10, 13, 62). Differentiation of the AF5 cell line requires no specific treatment other than allowing them to become confluent in growth media containing standard amounts (10%) of FCS. In serum-containing media, subconfluent exponentially growing cultures of the AF5 cells weakly expressed the GFAP astrocytic marker, while confluent cultures spontaneously differentiated into predominantly neuronal-like βIII-tubulin-expressing cells. Individual cells were positive for either GFAP or βIII-tubulin, but not both. BrdU experiments verified that few cells (ca. 1%) allowed to differentiate for 2 weeks were still dividing, as compared to almost all exponentially-growing cells. Additionally, nearly 80% of exponentially growing AF5 cells expressed the immature developmental marker nestin, while few or no cells expressing nestin could be found in differentiated cultures. Together these findings demonstrate developmental properties and phenotypic plasticity of this cell line that are consistent with properties described for neural progenitor cells.

The findings on changes in AF5 morphology and phenotype demonstrated that this cell line appears to be an excellent model for CNS cell differentiation, which could be further studied by microarray gene expression. Because AF5 cells were reversibly responsive to bFGF, we included bFGF-treated cells in the preliminary microarray analysis reported here. The relative homogeneity of cell lines, as opposed to other biological sources of RNA, make them ideally suited for gene expression profiling using microarray techniques. This consistency is indicated in the present study by the overall uniformity of the distribution of points along the linear regression axes in the scatter plots in Fig. 8.

The 15K-gene microarray is representative of all the unique human gene sequences that were available at the time the array was produced. Many of the genes with the highest intensity values (Z score > 8) in each of the conditions were ESTs or unnamed genes. A discussion of all the named genes that were increased or decreased in the three conditions is impractical within the context of this paper. However, selected genes that characterize this cell line under all three conditions are shown in Table 2, and Table 3 provides categories of genes that showed the greatest changes in the differentiated cells and the bFGF-stimulated cells as compared to normal growing cells. A variety of cellular functions are represented, including signaling, neurotransmision, structural elements and processes, protein processing and trafficing, cell cycle control and apoptosis, DNA function, chromosomes, transcription and translation, cell adhesion, growth and trophic factors, and general metabolic proteins and enzymes. The complete data can be accessed on the website to examine particular genes or gene sets of interest and develop comparisons as desired.

The following examples of gene expression support previous or anticipated results for AF5 cells in the different conditions (growing with and without bFGF stimulation, and differentiated). Markers for neurons (neurofilament) and astrocytes (GFAP) were expressed, confirming the immunostaining and Western blot results from the present and prior studies (52). Differences in expression of the bFGF receptor in the three conditions correlate well with the presence or absence of bFGF in the culture media, showing a highly significant increase in the bFGF cells (Z ratio $+2.17$). The cell-cycle protein E2F transcription factor 4, which binds the retinoblastoma family proteins p107 and p130 and is a key to cell cycle progression, was significantly expressed in the exponentially growing cells with and without bFGF (intensity value Z score $=$ 2.8 and 3.0, respectively), but was greatly decreased in the differentiated cells ($Z = 0$, Z ratio -3.23). Note that Z score and ratio values generally represent comparatively greater differences than traditional fold-differences, as the underlying data have been compressed by $\log_{10}$ transformation. Many of the genes with significant expression or changes in expression are known to be neural-tissue related; there were also products that are normally associated with hematopoetic and immune cells, perhaps illustrating the overlapping genetic programs that are shared among these cell types (17).

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