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ORIGINAL ARTICLE

Class III β-Tubulin Is Constitutively Coexpressed With Glial Fibrillary Acidic Protein and Nestin in Midgestational Human Fetal Astrocytes: Implications for Phenotypic Identity

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Abstract

Class III β-tubulin isotype (βIII-tubulin) is widely regarded as a neuronal marker in developmental neurobiology and stem cell research. To test the specificity of this marker protein, we determined its expression and distribution in primary cultures of glial fibrillary acidic protein (GFAP)-expressing astrocytes isolated from the cerebral hemispheres of 2 human fetuses at 18 to 20 weeks of gestation. Cells were maintained as monolayer cultures for 1 to 21 days without differentiation induction. By immunofluorescence microscopy, coexpression of βIII-tubulin and GFAP was detected in cells at all time points but in spatially distinct patterns. The numbers of $GFAP^+$ cells gradually decreased from Days 1 to 21 in vitro, whereas βIII-tubulin immunoreactivity was present in 100% of cells at all time points. Glial fibrillary acidic $protein^+$ and βIII-tubulin mRNA and protein expression were demonstrated in cultured cells by reverse-transcriptase-polymerase chain reaction and immunoblotting, respectively. Glial fibrillary acidic $protein^+$/βIII-tubulin-positive cells coexpressed nestin and vimentin but lacked neurofilament proteins, CD133, and glutamate-aspartate transporter. Weak cytoplasmic staining was detected with antibodies against microtubule-associated protein 2 isoforms. Confocal microscopy, performed on autopsy brain samples of human fetuses at 16 to 20 gestational weeks, revealed widespread colocalization of GFAP and βIII-tubulin in cells of the ventricular/subventricular zones and the cortical plate. Our results indicate that in the midgestational human brain, βIII-tubulin is not neuron specific because it is constitutively expressed in $GFAP^+$/$nestin^+$ presumptive fetal astrocytes.

Key Words: Astrocytes, Class III β-tubulin, Fetal glia, Glial fibrillary acidic protein, Microtubule associated protein 2, Nestin, Neural stem cells, Ventricular/subventricular zone

INTRODUCTION

The class III β-tubulin isotype (βIII-tubulin) is currently entrenched among investigators as a neuronal cell marker in the developing and mature human nervous system (1–5). The localization of this cytoskeletal protein after differentiation induction of multipotent human neural progenitors (6–8), embryonic stem cells (9, 10), hematopoietic/bone marrow-derived stem cells (11–14), adult skeletal muscle-derived mesenchymal stem cells (15, 16), and matrix cells from Wharton jelly (17) has been used to confirm the identity of neurons. Similarly, the detection of βIII-tubulin immunolocalization in primitive-appearing cells of the rostral migratory stream emanating from the subventricular zone (SVZ) of the anterior (frontal) horn, as well as in morphologically immature, proliferating subgranular cells of the hippocampus and the neocortex, has been construed as evidence in support of neurogenesis in the adult brain (18–24). The localization of βIII-tubulin in the SVZ in postnatal human brains has also been interpreted as neuronal specific (25), as has the expression of βIII-tubulin in bcl-2–immunoreactive "oligodendrocyte-like" cells normally present in the subventricular regions of the amygdaloid complex in human adult brains (26). At the same time, the specificity of cell markers with regard to the phenotypic identity of neurons derived from presumptive "multipotent" neural stem cells (NSCs) has been a subject of concern (27).

In the context of cancer, we and others have previously shown that βIII-tubulin is expressed in nonneuronal tumor phenotypes, including neoplastic astrocytes (28–30) and

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oligodendrocytes (31), in addition to being consistently present in differentiating neuronal phenotypes in bona fide neuronal/neuroblastic tumors or "primitive neuroectodermal tumors" with neuronal differentiation (4, 5, 32). Although βIII-tubulin is not expressed in nontransformed, differentiated/quiescent astrocytes and oligodendrocytes (4, 5), until now, the expression and cellular distribution of this so-called "neuron-specific" β-tubulin isotype has not been critically evaluated in the context of normal gliogenesis.

To test the specificity of this marker in divergent neuroepithelial cell lineages of the developing human telencephalon, we determined the expression of βIII-tubulin in relation to other cytoskeletal marker proteins, in primary cultures of glial fibrillary acidic protein (GFAP)-expressing fetal astrocytes isolated from the cerebral hemispheres of 2 human fetuses at 18 to 20 gestational weeks (g.w.). We then compared the immunoreactivity profiles of βIII-tubulin to those of GFAP and to the stem cell–associated intermediate filament protein nestin in autopsy brain sections derived from human fetuses of corresponding gestational ages. Our findings indicate that the βIII-tubulin is constitutively expressed, in conjunction with GFAP and nestin, in primary cultures of fetal astrocytes and in progenitor-like cells of the VZ/SVZ and the cortical plate in autopsy-derived histologic samples from the midgestational human brain. These data have both theoretic and practical implications in neurogenesis and gliogenesis underscoring potential problems of phenotypic identity involving morphologically immature cells of the developing human brain, which exhibit immunoreactivity for βIII-tubulin either in vitro and/or in situ.

MATERIALS AND METHODS

Primary Cultures of Human Fetal Astrocytes

Two primary culture sources for the study of human fetal astrocytes were used for comparison and to ensure data reproducibility. The first was generated in the laboratory of one of us (KK), and the second was commercially obtained.

Preparation of Primary Human Fetal Astrocyte Cultures

Primary astrocyte cultures were prepared from fresh brain tissue collected from 16 to 18 g.w. human fetuses. Fetal brain tissue was obtained with informed consent by Advanced Biosciences Resources, Inc. (Alameda, CA). Tissue was obtained with no identifiers under the approval of the institutional review board of Temple University. The procedure is based on the methods of Cole and de Vellis (33) and Yong and Antel (34), with additional modifications. The tissue was maintained overnight at 4°C in Hanks balanced salt solution containing gentamicin and amphotericin B. The tissue was washed in fresh cold Hanks balanced salt solution, and the blood vessels and any meninges were removed. Subsequently, the tissue was mechanically dissociated by mincing and repeated pipetting. The tissue was then enzymatically digested with 0.05% trypsin in Hanks balanced salt solution at 37°C with gentle shaking for 1 hour followed by trypsin inactivation by the addition of fetal bovine serum. The tissue was then passed through a 75-μm/L nylon mesh, and the resulting single-cell suspension was then plated at a density of 1 to 5 $\times$ 10^4 cells/cm^2 in Dulbecco's modified Eagle's medium-Ham F12 media containing 10% fetal bovine serum, 2 mmol/L of L-glutamine, 5 μg/ml of insulin, 100 μg/ml of gentamicin, and amphotericin B. Cells were refed 4 to 5 days after plating. After approximately 1 week, the primary mixed cultures were placed on a rotary shaker at 200 rpm in a 37°C incubator with 7% CO_2 for approximately 16 hours in serum media as described by Cole and de Vellis (33), and nonadherent cells were discarded. The astrocyte cultures were then refed with Dulbecco's modified Eagle's medium-Ham F12 media containing 15% fetal bovine serum and 100 μg/ml of gentamicin and were subcultured at a ratio of 1:4 after approximately 1 week, when confluence reached approximately 80%. The cells were then plated onto glass chamber slides for fixation and analysis. Primary cultures were "passage 2" at the time of plating.

Human Fetal Astrocytes

Proliferating nonimmortalized, nontransformed human fetal astrocytes, isolated from the cerebral hemisphere of an 18-g.w. human male fetus (Clonetics Astrocyte Cell Systems), were commercially purchased from Cambrex Bio Science (Walkersville, MD). The cells were maintained as monolayer cultures in tissue culture–treated flasks for 6 to 9 days in an astrocyte growth medium (CC-3186; Clonetics AGM BulletKit) containing Astrocyte basal medium (CCMD 190, Clonetics) and the following growth supplements: human recombinant growth factor, insulin, ascorbic acid, gentamicin-amphotericin B, L-glutamine, and 5% fetal bovine serum. The cells were harvested and cryopreserved before the primary cultures reached confluence and were analyzed at the first passage out of cryopreservation.

Other Cell Cultures

P19 mouse embryonal carcinoma cells were used as controls for βIII-tubulin and microtubule-associated protein 2 (MAP2) immunoreactivity in differentiating neuronal cells. P19 cells were cultured and differentiated by 1 μmol/L all *trans*-retinoic acid (Sigma-Aldrich, Prague, Czech Republic) as described previously (35). Briefly, cells in bacterial-grade Petri dishes were aggregated 4 days in a medium containing 1 μmol/L retinoic acid. The aggregates were then dispersed onto a single cell suspension. The suspension in medium without retinoic acid was replated on a culture dish containing glass coverslips. After 4 days, fresh medium supplemented with cytosine arabinoside (Sigma-Aldrich) at a concentration of 5 μg/ml was added to inhibit proliferating nonneuronal cells, whereas culturing continued for an additional 3 days.

The human glioblastoma cell line U118MG was maintained in Dulbecco's modified Eagle's media containing 4 mmol/L L-glutamine, 10% fetal bovine serum, and antibiotics as described previously (36). Mouse embryonic fibroblasts 3T3 cells, grown in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics, were used as nonneural controls. All cell lines were obtained from American Type Culture Collection (Manassas, VA).

Fetal Brain Sections

Formalin-fixed, paraffin-embedded autopsy tissue samples from the cerebral hemispheres at the level of the body of the lateral ventricles of 4 human male fetuses corresponding to 18 to 20 g.w. were studied for comparison as reference tissues from the intact brain. These specimens were devoid of neuropathologic abnormalities. The gestational age of the fetuses was estimated by the number of weeks after ovulation, crown-rump length, and other anatomic landmarks. The use of existing archival tissue material in the present study was subject to approval by an institutional review board exempt review (Drexel University IRB Protocol No. 17148). No patient identifiers were used.

Microtome sections from archived formalin-fixed, paraffin-embedded tissue blocks were cut at 5 μm in thickness, placed on electromagnetically charged slides, and stained with hematoxylin and eosin for morphologic evaluation. Adjacent, serially cut, and sequentially numbered sections were processed for immunohistochemistry and immunofluorescence microscopy.

Antibodies

For the detection of βIII-tubulin, 3 mouse monoclonal antibodies, TuJ1 (immunoglobulin [Ig]G2a), TU-20 (IgG1), and SDL.3D10 (IgG2b), directed against epitopes on the C-terminal end of the neuron-specific βIII-tubulin were used. The characterization, purification, and production of TuJ1 (Covance, Princeton, NJ) has been described elsewhere (37–39). Antibodies TU-20 (2) and SDL.3D10 (Sigma) were prepared against a conserved synthetic peptide from the C-terminus of the human βIII-tubulin. In addition, an affinity-purified rabbit antibody against the CMYEDDD-DEESEAQGPK peptide identical to the βIII carboxyl terminal, isotype-defining domain detected by monoclonal antibody TuJ1 was used (40). α-Tubulin was detected by mouse monoclonal antibody TU-01 (IgG1) (41, 42) conjugated with fluorescein isothiocyanate (FITC) (EXBIO, Prague, Czech Republic). β-Tubulin was detected by mouse monoclonal antibody TUB2.1 directly conjugated with indocarbocyanate (Cy3) (Sigma).

Glial fibrillary acidic protein was detected with mouse monoclonal antibodies GF-01 (IgG1) (43) or 6F2 (IgG1κ) (DAKO, Carpinteria, CA). For the immunophenotypic characterization of human fetal astrocyte cultures, the following monoclonal antibodies were used: VI-01 (IgM) (44) and VI-10 (IgM) (45) against vimentin, MT-01 (IgG1) (46) and MT-02 (IgM) (46) against MAP2ab and AP18 (IgG1) (47) against MAP2abc, anti-neurofilament protein (NF) antibodies NF-09 (IgG2a) against a nonphosphorylated epitope on NF-M (48) and NF-01 (IgG1) (49) against a phosphorylated epitope on NF-H, and monoclonal antibodies 10C2 (IgG1) (Chemicon, Temecula, CA) and 2C1 3B9 (IgG1) (Covance) against nestin were used. For further phenotypic characterization of human fetal astrocytes, a rabbit antibody against CD133 (a stem cell marker) (Abcam, Cambridge, MA) and a goat antibody against excitatory amino acid transporter/glutamate-aspartate transporter (GLAST) (a marker for radial glia; Santa Cruz Biotechnology, Santa Cruz, CA) were also used.

Cy3-Conjugated anti-mouse and FITC-conjugated anti-rabbit antibodies for multiple staining were commercially obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Tetrarhodamine isothiocyanate-conjugated anti-goat antibody was obtained from Santa Cruz Biotechnology. Fluorescein isothiocyanate-conjugated anti-mouse antibody and Texas Red-conjugated anti-rabbit antibody were bought from Vector Laboratories (Burlingame, CA). Peroxidase-conjugated anti-mouse antibody was obtained from Promega (Madison, WI).

Immunofluorescence on Human Fetal Astrocytes in Culture

Immunofluorescence microscopy on fixed cells was performed as described previously (50). Briefly, cells grown on coverslips were rinsed with microtubule stabilizing buffer (MSB) supplemented with 4% polyethylene glycol 6000, extracted for 2 minutes with 0.2% Triton X-100 in MSB, and fixed for 20 minutes in 3% formaldehyde in MSB. Cells were also fixed for 20 minutes in 3% formaldehyde in MSB and thereafter extracted for 4 minutes in 0.5% Triton X-100 in MSB. Alternatively, cells were only fixed for 20 minutes in 3% formaldehyde in MSB. Monoclonal anti-βIII-tubulin antibodies TuJ1 and SDL.3D10 were diluted 1:500 and 1:250, respectively. Antibody TU-20 was used as undiluted supernatant. The polyclonal antibody against βIII-tubulin was diluted 1:500. Monoclonal anti-GFAP antibody GF-01 was used as undiluted supernatant. Antibodies 10C2 and 2C1 3B9 against nestin were diluted 1:200, and antibody AP18, in the form of concentrated supernatant, was diluted 1:250. Monoclonal antibodies VI-01 and VI-10 against vimentin, MT-01 and MT-02 against MAP2ab, NF-01 against NF-H, and NF-09 against NF-M were used as undiluted supernatants. Antibodies against CD133 and GLAST were diluted 1:100, and 1:50, respectively. Anti-CD133 and anti-GLAST antibodies were used in formaldehyde-fixed cells to preserve immunoreactivity for cell-surface antigens. Cy3-conjugated anti-mouse antibody was diluted 1:500. Fluorescein isothiocyanate-conjugated anti-mouse and anti-rabbit antibodies, as well as tetrarhodamine isothiocyanate-conjugated anti-goat antibody, were diluted 1:100.

For double labeling of cells with 2 mouse monoclonal antibodies, the coverslips were incubated with the first antibody, followed by incubation with fluorochrome-conjugated anti-mouse antibody. The remaining binding sites on the anti-mouse antibody were blocked by incubation with normal mouse serum (diluted 1:10) prior to incubation with the second directly conjugated mouse antibody. Fluorescein isothiocyanate-conjugated TU-01 antibody against α-tubulin and Cy3-conjugated TUB2.1 antibody against β-tubulin were diluted 1:50. 4,6-diamidino-2-phenylindole was used to label cell nuclei. The preparations were mounted in MOWIOL 4-88 (Calbiochem, San Diego, CA) and examined with an Olympus A70 Provis microscope. Conjugates alone did not give any detectable immunoreactivity.

Counting of immunolabeled fixed cells grown on coverslips was performed manually. Ten nonoverlapping fields were counted for each coverslip, and 50 cells were counted in each field. The percentage of cells counted for

each marker protein was calculated at 1, 4, 7, 8, 11, 17, and 21 days after plating as monolayer cultures.

Immunohistochemistry and Immunofluorescence on Fetal Brain Sections

Deparaffinized histologic sections from archival formalin-fixed, paraffin-embedded fetal brain tissues were used for immunoperoxidase and immunofluorescence studies as described previously (30). For immunohistochemical studies, we used the avidin biotin complex peroxidase method according to the manufacturer's instructions (Vector Laboratories). Briefly, the modified protocol included deparaffinization in xylene, rehydration in graded alcohols up to water, nonenzymatic antigen retrieval in citrate buffer pH 6.0, and quenching of endogenous peroxidase with 10% H_2O_2 in methanol. After rinsing with PBS, sections were blocked with normal horse serum for 1 hour and incubated with primary antibodies at room temperature in a humidified chamber overnight. Primary mouse monoclonal antibodies included anti-βIII-tubulin (clone TuJ1; 1:500 dilution), anti-GFAP (clone 62F; 1:100), and anti-nestin (clone 2C1 3B9; 1:250 dilution). After thorough rinsing with PBS, biotinylated anti-mouse secondary antibodies were incubated for 1 hour, followed by avidin biotin peroxidase complexes (ABC Elite Kit; Vector Laboratories). Finally, the peroxidase was revealed with diaminobenzidine (Roche, Basel, Switzerland), and sections were counterstained with hematoxylin and coverslipped with Permount. Negative controls included omission of primary antibody and substitution with nonspecific mouse IgG1 and IgG2b, which were used as Ig class-specific controls (corresponding to the Ig subclasses of the primary antibodies used in this study; Becton Dickinson, Franklin Lakes, NJ). Experiments using nonconjugated isotype-matched control monoclonal antibodies did not show any nonspecific binding of the secondary rabbit anti-mouse IgG1 and IgG2b antibodies. For the evaluation of immunoperoxidase staining, an Olympus AX70 bright-field microscope equipped with a DP12 digital camera (Olympus) was used.

For double labeling immunofluorescence studies on deparaffinized sections, we used the same 3 antibodies at the same dilutions as described above. After incubation with the first primary antibody, an FITC-conjugated anti-mouse antibody was incubated for 2 hours, followed by the second primary antibody overnight, and a second Texas Red-tagged secondary antibody was incubated for 2 hours. 4,6-diamidino-2-phenylindole was used to label cell nuclei. Slides were coverslipped using an aqueous-based mounting medium (Vectashield Hard Set; Vector Laboratories). For immunofluorescence microscopy, an inverted Nikon fluorescence microscope (Eclipse 300; Nikon, Tokyo, Japan) with a digital camera from Q Imaging (model Retiga 1300) and deconvolution software (Slidebook 4.0, Intelligent Imaging, Denver, CO) were used.

Immunolabeled sections were also evaluated with a Leica TCS SP2 AOBS (Acousto-Optical Beam Splitter) laser confocal system equipped with a Leica DMRE fluorescence microscope. This system is outfitted with a single-prism spectrophotometer and 4 sets of movable slit in front of the detection photomultiplier used for the detection of fluorescence emission and minimization of crosstalk. For balanced excitation of the fluorochromes, the lasers are combined with acoustico-optical tunable filter system, which enables to adjust the individual intensity of the 3 laser lines (argon, 488 nm; green neon, 543 nm; and helium neon, 633 nm) independently. A diode (UV laser, 405 nm) is also part of this system. 4,6-diamidino-2-phenylindole was excited by a 405-nm beam using a neutral density filter of 12.5 and was detected through a spectral of 410 to 482 nm (emission peak of 456 nm); FITC was excited by a 488-nm laser beam and was detected through a spectral range of 500 to 538 nm (emission peak of 520 nm); Texas Red was excited by the 543-nm beam and was detected through a spectral range of 589 to 713 nm (emission peak of 620 nm).

Gel Electrophoresis and Immunoblotting

Whole-cell extracts for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were prepared by solubilizing washed cells in hot sodium dodecyl sulfate sample buffer and boiling for 5 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.5% gel, electrophoretic transfer of separated proteins onto nitrocellulose, and details of the immunostaining procedure are described elsewhere (50). The monoclonal antibody TuJ1 against βIII-tubulin was diluted 1:2000, and monoclonal antibodies GF-01 against GFAP and NF-09 against NF-M were used as undiluted supernatants. Secondary anti-mouse antibody conjugated with peroxidase was diluted 1:10000. The binding was detected with chemiluminescence reagents (Pierce, Rockford, IL) in accordance with the manufacturer's directions.

RNA Isolation and Reverse-Transcriptase–Quantitative Polymerase Chain Reaction Analysis

Total cellular RNA was extracted from cells by the RNeasy Mini kit according to the manufacturer's directions (Qiagen, Valencia, CA). The purity and integrity of the RNA preparations were checked spectroscopically and by 1% formaldehyde-agarose gel electrophoresis. One microgram of total RNA in a 20-μl reaction mixture was converted to cDNA using the ImProm-II reverse transcription kit (Promega) with oligo(dT) primer according to the manufacturer's instructions. Twenty μl of the cDNA reaction mixture was diluted 5 times in diethyl pyrocarbonate–treated water to prevent inhibition of Taq polymerase in subsequent polymerase chain reaction (PCR) reaction. One microliter of diluted cDNA product was used in each PCR reaction. Amplifications were performed in 10-μl PCR reaction mixtures containing QuantiTect SYBR Green PCR Master Mix (Qiagen) and 0.5 μmol/L of each human gene-specific primers for βIII-tubulin (TUBB3; NCBI Refseq ID NM_006086) and β-actin (ACTB, NCBI Refseq ID NM_001101). Primer sequences were as follows: βIII-tubulin forward 5′-GCGAGATGTACGAAGACGAC-3′, reverse 5′-TTTAGACACTGCTGGCTTCG-3′ (51); and β-actin forward 5′-TCCTTCCTGGGCATGGAGT-3′, reverse 5′-AAAGCCATGCCAATCTCATC-3′. Oligonucleotides

were synthesized by East Port (Prague, Czech Republic). Real-time quantitative PCRs were carried out on Mastercycler realplex (Eppendorf, Wesseling-Berzdorf, Germany). Amplification started with a 15-minute initial activation step at 95°C, followed by 50 cycles of 15-second denaturation at 94°C, 20-second annealing at 60°C, and 10- to 20-second (according to length of the product) extension at 72°C. Fluorescence was determined during each elongation step. At the end of each run, melting curve analysis was performed to ascertain the presence of a single amplicon. Standard curve for each PCR run was generated by 3-fold serial dilution of sample cDNA. Quantification was performed using the Realplex software version 1.5 (Eppendorf) and applying second-derivative maximum method. Experiments were performed twice with triplicate samples (cDNA isolated from 3 separate cultures). The expression of analyzed genes was normalized to expression of β-actin. Nontemplate (without cDNA) and non-reverse-transcriptase (10 ng of total RNA instead of cDNA) control reactions were performed in each PCR run. To ensure that correct DNA fragments were amplified, the size of all PCR products was controlled by electrophoresis. Statistical analysis was performed with the Student unpaired *t*-test.

RESULTS

Class III β-tubulin, GFAP, and Nestin are Coexpressed in Human Fetal Astrocytes in Culture

Primary cultures were "passage 2" at the time of plating, whereas all primary cultures examined in the present study were passaged no more than 4 times. After extraction with Triton X-100 followed by fixation with formaldehyde, all 3 anti-βIII-tubulin monoclonal antibodies rendered microtubule staining by immunofluorescence microscopy. The intensity and spatial distribution of microtubule staining were conspicuously greater with antibody TuJ1 as compared with TU-20 and SDL.3D10 (Fig. 1). Specifically, monoclonal antibody TuJ1 rendered a more robust and generalized staining pattern, decorating microtubules, which emanated from microtubule-organizing center and reached the periphery of the cytoplasm (Fig. 1A). In comparison, TU-20 (Fig. 1B) and SDL.3D10 (Fig. 1C) gave dot-like staining of microtubule arrays. The staining pattern of the anti-βIII-tubulin antiserum was identical to that of TuJ1 (not shown). The microtubule-associated localization of βIII-tubulin was further demonstrated by double labeling with antibody TU-01 against α-tubulin (Fig. 2A) and antibody TU-20 (Fig. 2B). A similar staining pattern was obtained in cells fixed in cold methanol and acetone without exposure to Triton X-100 extraction (not shown).

Double labeling using antibodies to βIII-tubulin and GFAP as well as antibodies to βIII-tubulin and nestin revealed coexpression but differential spatial patterns of cytoskeletal localization by immunofluorescence microscopy (Fig. 3). Morphologically, $GFAP^+$/$\beta III\text{-tubulin}^+$ cells from both astrocyte culture sources and all time points (from 1 to 17 days in vitro) exhibited mixed flat/apolar and fibrillated/multipolar features (Figs. 3A–C). Ostensibly, at low magnification, cells with multipolar processes gave the appearance of a preponderance of GFAP staining as compared with flat apolar cells; however, both multipolar and apolar cells were $\beta III\text{-tubulin}^+$ (Figs. 3A–C).

Class III β-tubulin, GFAP, and nestin exhibited distinct fibrillar cytoskeletal localizations (Figs. 3A–L). Class III β-tubulin staining was associated with microtubule arrays (Figs. 3G, J), whereas GFAP (Fig. 3H) and nestin (Fig. 3K) staining was filamentous and distinct from that encountered with anti-βIII-tubulin antibodies (Figs. 3I, L). Prominent βIII-tubulin and GFAP staining was also detected in the perinuclear cytoplasmic region because microtubules and glial intermediate filaments originate from this region (Figs. 3G–I). In addition, strong filamentous vimentin staining was present (not shown).

When analyzed at the first passage out of cryopreservation, more than 90% of cells stained positive for GFAP. From Days 1 to 7 in vitro, approximately 95% of cells were $GFAP^+$, followed by a decline of GFAP immunoreactivity in approximately 90%, 80%, 60%, and 25% of cells at Days 8, 11, 17, and 21, respectively. Although the numbers of $GFAP^+$ cells were found to gradually decrease in vitro, no change in the numbers of $\beta III\text{-tubulin}^+$ cells was noted, as documented by double label staining for βIII-tubulin and GFAP at Day 21 (Figs. 3D–F). βIII-Tubulin was present in 100% of cells at all time points.

Diffuse and very weak cytoplasmic immunoreactivity was detected with antibodies to MAP2ab and MAP2abc. Figure 4 exemplifies the divergent staining in fetal astrocytes obtained with antibody AP18 against MAP2abc (Fig. 4A) and anti-βIII-tubulin antibody (Fig. 4B). By comparison, P19 embryonal carcinoma cells, stimulated to undergo neuronal differentiation by retinoic acid, exhibited robust staining in

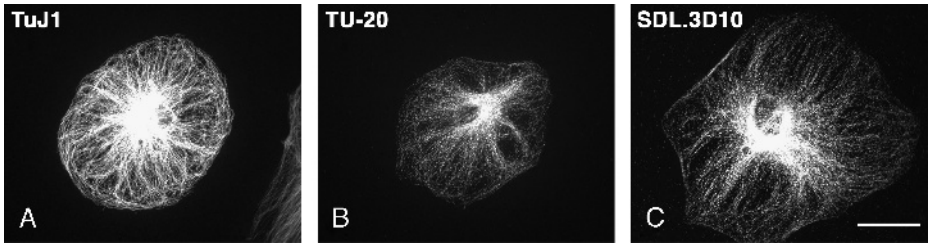

FIGURE 1. Immunofluorescence staining of human fetal astrocytes maintained for 7 days in vitro with monoclonal antibodies against βIII-tubulin. TuJ1 **(A)**, TU-20 **(B)**, and SDL.3D10 **(C)**. Scale bar = 50 μm.

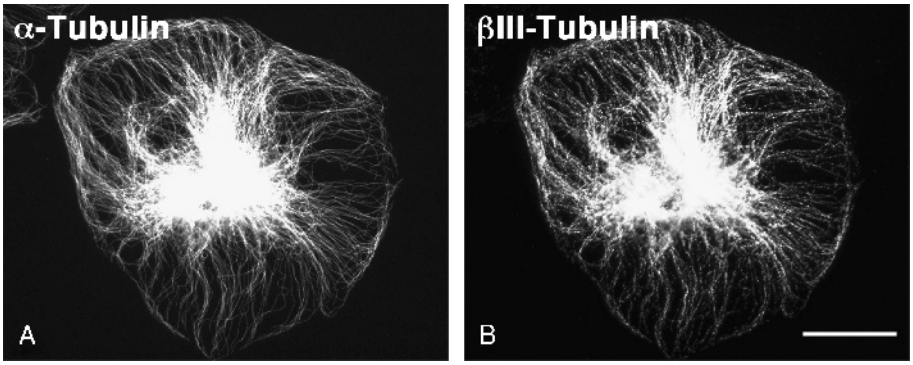

FIGURE 2. Codistribution of α-tubulin and βIII-tubulin demonstrated by immunofluorescence microscopy in primary culture of human fetal astrocytes maintained for 7 days in vitro. Cells were stained by double labeling with monoclonal antibody against α-tubulin directly labeled with fluorescein isothiocyanate **(A)** and monoclonal antibody TU-20 against class III β-tubulin isotype **(B)**. Scale bar = 50 μm.

the cell bodies and neurite-like cell processes both with AP18 (Fig. 4C) and anti-βIII-tubulin antibodies (Fig. 4D).

No immunoreactivity on fetal astrocytes was detected with antibodies against phosphorylated NF-H (antibody NF-01) and nonphosphorylated NF-M (antibody NF-09) epitopes on NFs, as well as the neural stem marker CD133 and the radial glial marker GLAST (see the Table for an overview of immunofluorescence profiles of cytoskeletal proteins in cultured human fetal astrocytes that reflects staining in 100% cells except GFAP). Control 3T3 cells exhibited staining of microtubule arrays cells with the antibody to α-tubulin; however, no GFAP, βIII-tubulin, or MAP2 staining was detected (not shown).

To confirm expression of mRNA for βIII-tubulin in fetal astrocytes, real-time reverse-transcriptase–quantitative PCR analysis was performed. Human glioblastoma cell line U118MG expressing βIII-tubulin (B. Šmejkalová and P. Dráber, unpublished observations) was used as positive control. The mRNA expression of βIII-tubulin in fetal astrocytes relative to that of U118MG cells is shown in Figure 5. Slightly higher expression was detected in fetal astrocytes (1.5 ± 0.1–fold; mean ± SEM; $n = 6$; $p < 0.05$). Immunoblotting experiments on whole cell lysates of fetal astrocytes also clearly demonstrated that both βIII-tubulin (Fig. 6; lane 1) and GFAP (Fig. 6; lane 2) were expressed in these cells, whereas NF-M was not detected by the NF-09 antibody (Fig. 6; lane 3).

Colocalization of GFAP, βIII-Tubulin, and Nestin in VZ/SVZ, Internal Capsule, and Cortical Plate of Midgestational Human Fetuses

By immunohistochemistry performed on autopsy brain samples of human fetuses 16 to 20 g.w., there was extensive staining of GFAP, βIII-tubulin, and nestin in populations of small/apolar, morphologically immature, progenitor-like cells in the VZ/SVZ in the ganglionic eminence at the level of the body of the lateral ventricles (Fig. 7). Given the high cellular density and close cellular apposition, as well as the dense βIII-tubulin labeling among progenitor-like cells in the SVZ, it was difficult to determine the proportion of cells that did not express βIII-tubulin (Fig. 7; inset).

By immunofluorescence microscopy, extensive colocalization of GFAP, βIII-tubulin, and nestin was demonstrated in cells of the VZ/SVZ (Fig. 8) in addition to subpopulations of individual cells expressing solely GFAP, βIII-tubulin, or nestin (Fig. 8F; inset and L). The widespread coexpression of GFAP and βIII-tubulin in the SVZ (germinal matrix) of the ganglionic eminence was confirmed by confocal microscopy using serial cell slicing and 3-dimensional maximum projection (Figs. 8G–I). Glial fibrillary acidic protein, βIII-tubulin, and nestin staining were also present in immature ependymal cells of the VZ at the body of the lateral ventricle as demonstrated by immunoperoxidase (Fig. 7) and immunofluorescence microscopy (Figs. 8D–F; arrowheads). In addition, GFAP and nestin colocalizations were demonstrated in radial fibers originating from cells of the ventricular surface consistent with basal processes of immature ependymal cells, rather than actual radial glia fibers (Figs. 8L, O; arrows).

In the region of the internal capsule, prominent GFAP and nestin immunolabeling was present in cells with short and slender fibrils, consistent with presumptive fetal astrocytes (Fig. 7; see insets corresponding to GFAP- and nestin-stained cells). In contrast, βIII-tubulin staining in the same region was less robust and was detected either in axon fibers or in the scanty perinuclear cytoplasm of small-sized cells exhibiting glial-like morphological features (Fig. 7; see inset corresponding to βIII-tubulin-stained cells).

In the telencephalic cortical plate, variable immunoperoxidase staining was detected for GFAP, βIII-tubulin, and nestin (Figs. 7, 8). Immunoreactivity for βIII-tubulin was abundant, making it difficult to accurately discriminate by immunohistochemistry between cell body and neuropil labeling (Fig. 7). By immunofluorescence microscopy, individual cells exhibited immunoreactivity for either of GFAP and βIII-tubulin (Fig 8C; inset), although colocalization of both proteins was also present in many cells of the developing cortical plate (Figs 8A–C), which was further corroborated by confocal microscopy (not shown).

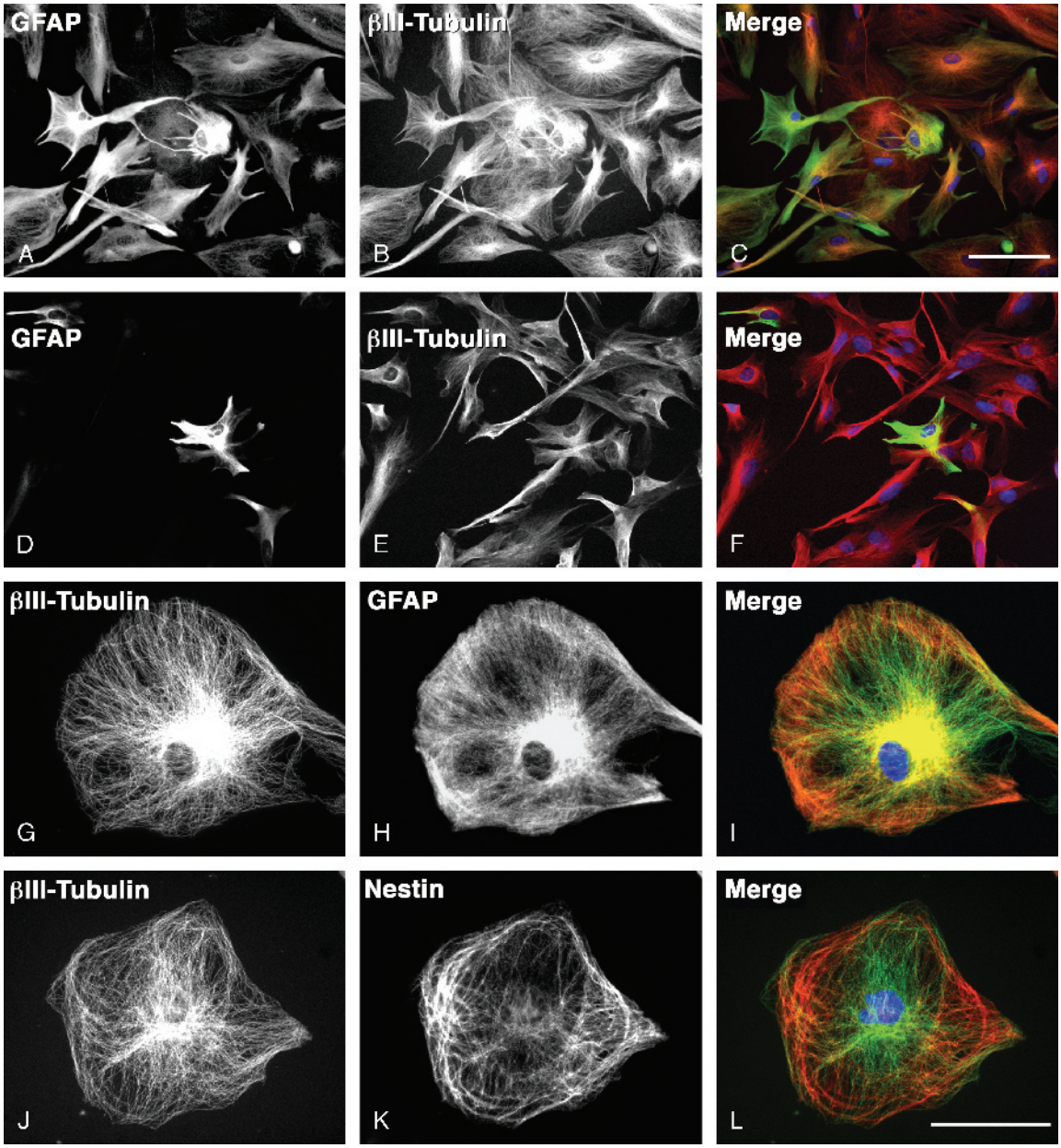

FIGURE 3. Distribution of glial fibrillary acidic protein (GFAP), class III β-tubulin isotype (βIII-tubulin), and nestin in primary cultures of human fetal astrocytes maintained as monolayers for 7 to 8 days or 21 days in vitro. **(A–C)** Panels depict cells (8 days in vitro) stained by double labeling with a monoclonal antibody against GFAP (**A**; green) and a polyclonal antibody against βIII-tubulin (**B**; red). **(C)** Superposition is shown. **(D–F)** Distribution of GFAP and βIII-tubulin in primary cultures of human fetal astrocytes maintained as monolayers for 21 days in vitro. Panels depict cells stained by double labeling with a monoclonal antibody against GFAP (**D**; green) and a polyclonal antibody against βIII-tubulin (**E**; red). **(F)** Superposition is shown. 4,6-diamidino-2-phenylindole (blue) labels nuclei (**F**). **(G–I)** Double labeled cells (7 days in vitro) for βIII-tubulin (**G**; green) and GFAP (**H**; red) at a higher magnification. **(I)** Superposition is shown. **(J–L)** Panels depict cells (7 days in vitro) double stained with a polyclonal antibody against βIII-tubulin (**J**; green) and a monoclonal antibody against nestin (**K**; red). **(L)** Superposition is shown. 4,6-diamidino-2-phenylindole (blue) labels nuclei **(C, F, I, L)**. Note the overlapping of βIII-tubulin and GFAP staining in paranuclear cytoplasmic region from where microtubules and glial intermediate filaments emanate (**G–I**; yellow). Scale bars = **(A–F)** 100 μm; **(G–L)** 50 μm.

DISCUSSION

Unexpected Expression of Class III β-Tubulin Isotype in Human Fetal Astrocytes

In this study, we have demonstrated expression of βIII-tubulin protein and mRNA in $GFAP^+$/$nestin^+$ astrocytes isolated from the midgestational human fetal cerebrum and maintained in cell culture for 1 to 21 days without further passage or differentiation induction. We have also determined that the distribution of βIII-tubulin immunoreactivity is ubiquitous, involving both flat/apolar and fibrillated/multipolar $GFAP^+$/$nestin^+$ astrocytes, exhibiting a cytoskeletal compartmentalization associated with microtubule arrays. The numbers of $GFAP^+$ cells were found to gradually decrease from Days 1 to 21 in vitro, whereas βIII-tubulin immunoreactivity was present in 100% of cells at all time points. In addition, we have demonstrated by immunohistochemistry and confocal microscopy that βIII-tubulin is coexpressed with GFAP and nestin in populations of progenitor-like cells in the telencephalic VZ/SVZ at the ganglionic eminence and the developing cortical plate. The coexpression of βIII-tubulin and GFAP in midgestational normal human fetal astrocytes maintained in primary culture is indeed an intriguing and hitherto underappreciated finding, the βIII-tubulin isotype being widely used as a bona fide neuronal marker (reviewed in [4, 5]). Rieske et al (52) have recently reported evidence of coexpression of βIII-tubulin, GFAP, and nestin in human fetal astrocytes (NHA, Clonetics) encountered after 3 passages under basal growth conditions; they assert that these cells can undergo divergent differentiation to become either neurons or astrocytes as a result of exogenous differentiation induction. Our findings confirm and extend the colocalization of βIII-tubulin and GFAP reported in the latter study (ibid) in the following respects: first, the coexpression of βIII-tubulin, GFAP, and nestin was confirmed in 2 primary astrocyte cultures from different sources, one of which was also used in the study by Rieske et al (52), but neither of which was passaged more than 4 times. Second, in the present study, the coexpression of GFAP and βIII-tubulin was also confirmed at the mRNA level. Finally, the present study elucidated distinct patterns of cytoskeletal localization in the cytoplasm of cultured cells coexpressing these molecules. In all, the progressive decline of GFAP immunoreactivity compared with the unchanged immunolabeling for βIII-tubulin in cells maintained for up to 21 days in vitro argues in favor of a constitutive expression of βIII-tubulin in human fetal astrocytes, rather than an acquired cytoskeletal feature associated with multiple cell passages.

The coexpression of βIII-tubulin, GFAP, and nestin in cultured human astrocytes (but also in progenitor-like cells in the SVZ and in cells of the developing cortical plate) may be consistent with a "multidifferentiated" phenotype in the context of NSC properties of SVZ and cortical astrocytes (19, 53–57). Whereas the widespread coexpression of βIII-tubulin, GFAP, and nestin in the midgestational human VZ/SVZ reported in the present study is, to our knowledge, a

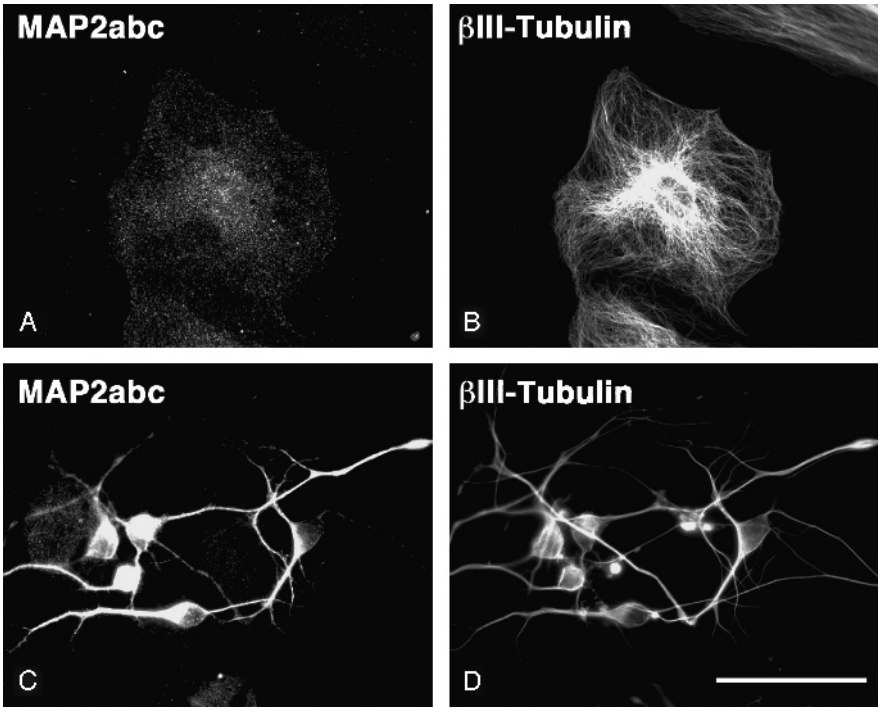

FIGURE 4. Differential distribution of microtubule-associated protein 2 (MAP2)abc and class III β-tubulin isotype (βIII-tubulin) in primary cultures of human fetal astrocytes **(A, B)** and in P19 murine embryonal carcinoma cells showing neuronal differentiation after exposure to retinoic acid **(C, D)**. Note faint diffuse cytoplasmic staining for MAP2abc **(A)** as compared with prominent microtubule staining for βIII-tubulin **(B)** in human fetal astrocytes maintained for 7 days in vitro. In contrast, neuronally differentiating P19 embryonal carcinoma cells exhibit robust staining in the cell bodies and neurite-like cell processes for both MAP2abc **(C)** and βIII-tubulin **(D)**. Scale bar = 50 μm.

TABLE. Summary of Cytoskeletal Protein Immunofluorescence in Human Fetal Astrocytes in Primary Culture*

Antibody	Antigen	Immunoreactivity
TU-20	βIII-tubulin	++ microtubules
SDL 3D10	βIII-tubulin	++ microtubules
TUJ 1	βIII-tubulin	+++ microtubules
Anti-βIII-tubulin polyclonal	βIII-tubulin	+++ microtubules
TU-01	α-tubulin	+++ microtubules
GF-01	GFAP	+++ filaments
6F2	GFAP	+++ filaments
VI-01	Vimentin	++ filaments
VI-10	Vimentin	++ filaments
10C2	Nestin	+++ filaments
2C1 3B9	Nestin	+++ filaments
MT-01	MAP2ab	+/− diffuse
MT-02	MAP2ab	+/− diffuse
AP-18	MAP2abc	+/− diffuse
NF-09	NF-M (P−)	Absent
NF-01	NF-H (P+)	Absent

*Data summarize immunoreactivity at 7 days in vitro. Immunoreactivity is rated as +++, strong; ++, moderate; +, weak; and +/−, very weak.

GFAP, glial fibrillary acidic protein, MAP2, microtubule-associated protein 2, NF-H, neurofilament protein high molecular weight isoform, NF-M, neurofilament protein middle molecular weight isoform, P+, phosphorylated, P−, nonphosphorylated.

novel finding, Mo et al (58) recently demonstrated coexpression of these cytoskeletal proteins in rare cells of the VZ/SVZ in a 9-g.w. human fetus. Similarly, a study by Itoh et al (59) has demonstrated that nestin-expressing neurospheres, isolated from $GFAP^+$ astrocytes of the cerebral cortex, are capable of differentiating into $\beta III\text{-tubulin}^+/GFAP^+/A2B5^+$ cells after 4 days of culture without exposure to β fibroblast growth factor and epidermal growth factor, indicating that cerebral cortical astrocytes may possess bipotential properties. Along these lines, Strathmann et al (60) have also

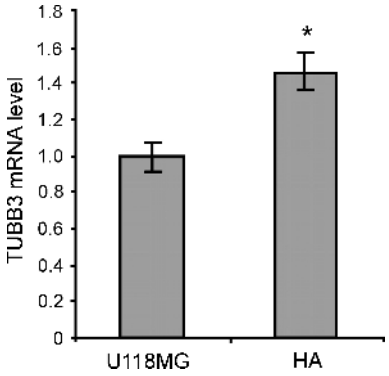

FIGURE 5. Transcription of *TUBB3* gene in fetal astrocytes relative to the level in glioblastoma cells U118MG. For 8 days in vitro, human fetal astrocytes (HAs) were maintained as monolayers. The expression was normalized to expression of β-actin. Data are presented as the mean fold change ± standard error of mean obtained from 2 independent experiments with triplicate samples (cDNA isolated from 3 separate cultures). *, Mean values significantly differs from U118MG: $p < 0.05$.

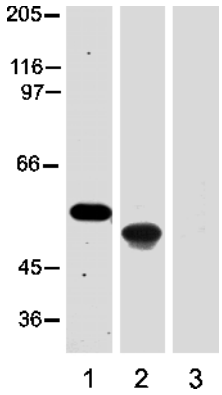

FIGURE 6. Immunoblot analysis of whole cell extracts from human fetal astrocytes maintained 7 days in culture. Staining with antibody TuJ1 to class III β-tubulin isotype (βIII-tubulin; lane 1), GF-01 to glial fibrollary acidic protein (lane 2), and NF-09 to NF-M (lane 3). The same amount of protein (10 μg) was loaded into each lane. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

recently identified 2 novel glial-restricted populations in the embryonic telencephalon, 1 of which exhibits an $A2B5^+/\beta III\text{-tubulin}^+$ putative astrocytic phenotype.

The coexpression of βIII-tubulin, GFAP, and nestin in cultured astrocytes, and also in cells of the intact fetal brain, bears certain similarities to the antigenic phenotype of radial glia. In particular, the βIII-tubulin has been shown to be present in GFAP-expressing radial glial cells although at an earlier gestational stage (61). The expression of βIII-tubulin in conjunction with MAP2 and phosphoneurofilaments in a subpopulation of GFAP-expressing radial glia has been construed as evidence of neuronal-glial bipotentiality (61). Radial glia are considered to have NSC properties (58, 61–63). Similarly, βIII-tubulin expression has also been reported in Müller glia cell lines, where retinal Müller glia (like telencephalic radial glia) have been assigned the role of NSCs (64). In light of the previously discussed topic, the unexpected localization of βIII-tubulin in GFAP-expressing cells isolated from the cerebral hemisphere of second-trimester human fetuses indicates that fetal astrocytes may be endowed with NSC properties capable of bipotential differentiation into neurons and glia (52, 59). Thus, a central issue pertaining to primary cultures from fetal human brain relates to the identity of $GFAP^+$ cells, which are conventionally defined as "astrocytes." However, because GFAP is also expressed by radial glia and "neurogenic" cortical astrocytes, these cultures may also potentially be regarded as NSC cultures.

Because radial glia of the human telencephalon persist through 18 to 20 g.w. (65), we examined the possibility that the fetal astrocytes used in the present study can represent morphologically modified cells exhibiting antigenic features of radial glia. Our data indicate that despite the coexpression of GFAP, βIII-tubulin, and nestin in the cultured fetal astrocytes, the lack of expression of GLAST and phosphoneurofilaments coupled with only a very weak staining with antibodies to MAP2ab (MT-01, MT-02) and MAP2abc (AP18), which was not associated with microtubules, does

not mirror the immunohistochemical profile of human radial glia as previously reported by Zecevic (61). It should be noted that excitatory amino acid transporter (GLAST) is enriched in the proliferative zones and radial glia in 13- to 20-g.w. human fetuses (66). Moreover, the lack of CD133 immunoreactivity in cultured fetal astrocytes raises questions as to whether these cells represent NSCs. CD133 is a marker for embryonic NSCs, an intermediate radial glial/ependymal cell type in the early postnatal stage, and for ependymal cells in the adult brain, but is absent in putative neurogenic astrocytes in the adult SVZ (67).

An alternative interpretation is that the coexpression of βIII-tubulin in GFAP$^+$ fetal brain cells may reflect developmentally immature astrocytes exhibiting a hitherto unknown transient, nonneuronal expression of βIII-tubulin in the context of gliogenesis (4, 5) in a manner analogous to the transient MAP2 expression in immature human glia (68). Along these lines, MAP2-like staining has been previously reported in nascent astrocytes derived from epidermal growth factor-generated murine neuroprogenitor cells (69), whereas MAP2c expression has been clearly documented in epidermal growth factor-responsive precursors in the context of gliogenesis (70). Similar to the unexpected pattern of βIII-tubulin/GFAP coexpression encountered in the present study, Rosser et al (70) noted that at early time points of epidermal growth factor-induced NSC differentiation, most GFAP$^+$ cells were also MAP2c$^+$, suggesting that this may represent a normal stage of astroglial development. Therefore, the possibility that βIII-tubulin expression in cultured astrocytes may merely represent a cytoskeletal adaptation of microtubules to cell culture conditions, as previously postulated in retinal epithelial cells (71), seems less tenable.

To our knowledge, the coexpression GFAP, βIII-tubulin, and nestin in immature ependymal cells of the VZ in the ganglionic eminence is a novel finding. The proliferative VZ persists up to 22 g.w. in humans, whereas extensive expression of GFAP has been shown in fetal ependymal cells of the lateral ventricle (72). In addition, the present study reveals for the first time in the human fetal brain colocalization of GFAP and nestin in radial fibers originating from VZ

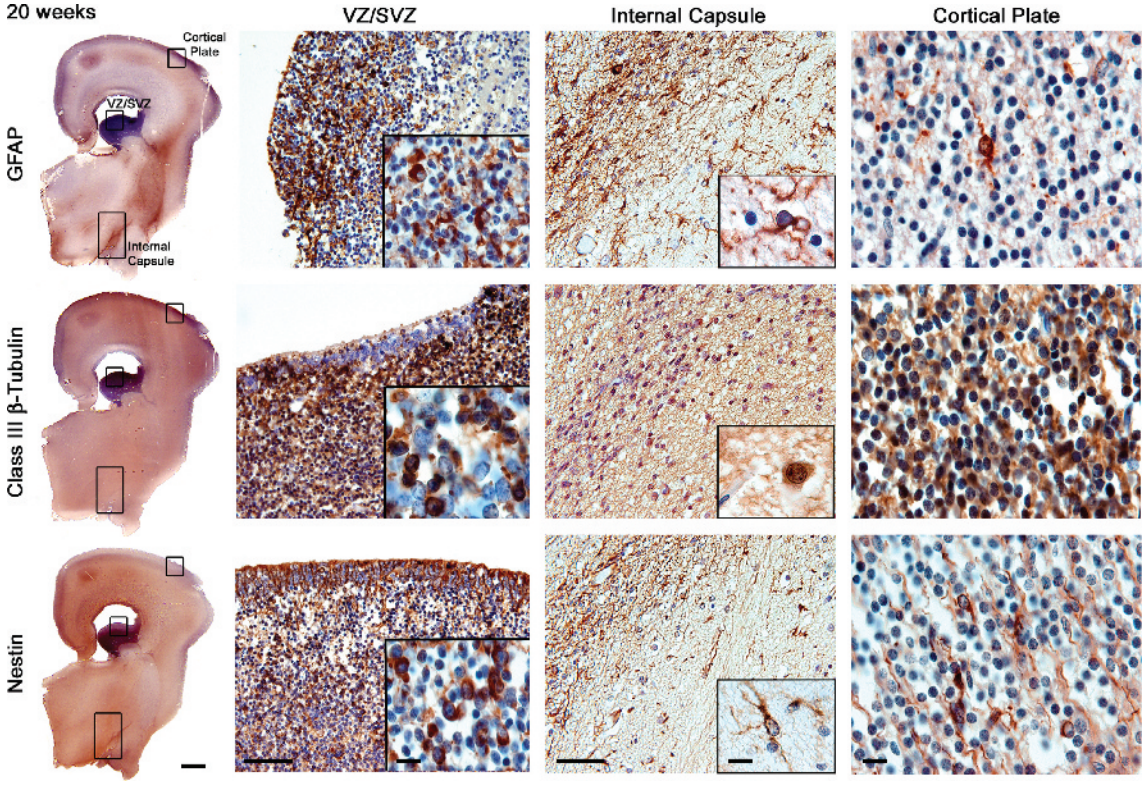

FIGURE 7. Immunohistochemical localizations of glial fibrillary acidic protein (GFAP), class III β-tubulin isotype (βIII-tubulin), and nestin in the ventricular and subventricular zones (VZ/SVZ), internal capsule, and cortical plate in immediately adjacent coronal sections of the right cerebral hemisphere of a 20-g.w. human fetus at the level of the ganglionic eminence. Note extensive staining of GFAP, βIII-tubulin, and nestin in populations of small/apolar, progenitor-like cells in the VZ/SVZ at the level of the body of the lateral ventricles (see insets). In addition, note prominent GFAP and nestin immunoreactivity in the region of the internal capsule where immunolabeled cells have short and slender fibrils consistent with presumptive fetal astrocytes (see insets corresponding to GFAP- and nestin-stained cells). Class III β-tubulin isotype staining in the internal capsule is less pronounced and is detected either in axon fibers or in the perinuclear cytoplasm of small-sized cells exhibiting glial-like morphology (see inset corresponding to βIII-tubulin-stained cells). Note the variable immunoreactivity for GFAP, βIII-tubulin, and nestin in the cortical plate. Class III β-tubulin isotype immunoreactivity is diffuse, making it difficult to discriminate between perikaryal and neuropil labeling. Avidin-biotin complex peroxidase with hematoxylin counterstain. Scale bar under coronal section of the fetal cerebral hemisphere, 500 μm; VZ/SVZ and internal capsule, 50 μm; cortical plate and insets, 10 μm.

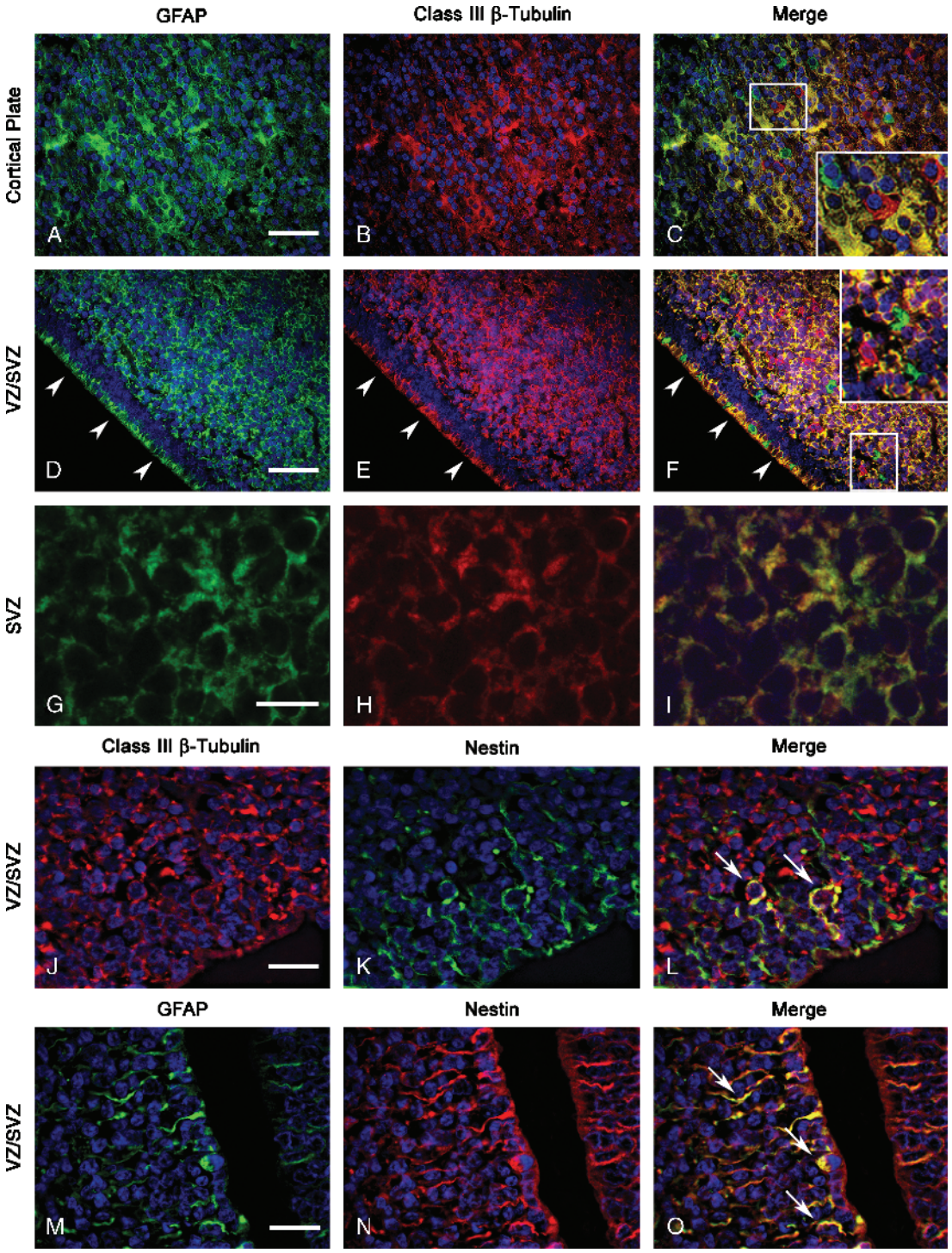

FIGURE 8. Distribution of glial fibrillary acidic protein (GFAP), class III β-tubulin isotype (βIII-tubulin), and nestin in the ventricular and subventricular zones (VZ/SVZ) and cortical plate of a 20-g.w. fetus as determined by immunofluorescence microscopy. Note localization of GFAP (**A**; green) and βIII-tubulin (**B**; red) in cells of the cortical plate. Colocalization of both proteins is depicted in yellow, in addition to individual cells expressing solely GFAP (green) or βIII-tubulin (red; **C**; see inset). In addition, note widespread expression of GFAP (**D**; green) and βIII-tubulin (**E**; red) in the VZ/SVZ. Coexpression is depicted in yellow in the merged image (**F**; see inset) and is confirmed by confocal microscopy using 3-dimensional maximum projection **(G–I)**. Note distribution of βIII-tubulin (**J**; red) and nestin (**K**; green) in the VZ/SVZ with colocalization of both proteins (**L**; arrows). Glial fibrillary acidic protein (**D**; green) and βIII-tubulin (**E**; red) staining is present in the VZ (**D–F**; arrowheads), whereas GFAP (**M**; green) and nestin (**N**; red) colocalizations are demonstrated in basal processes of immature ependymal cells in the VZ (**O**; arrows). Scale bars = **(A, D)** 50 μm; **(G, J)** 20 μm.

cells. These fibers are akin to basal processes of immature ependymal cells as opposed to radial glia fibers per se (72). Rodriguez-Perez et al (73) have previously shown that GFAP, vimentin, and nestin are coexpressed in the basal processes of mature bovine ependymal cells lining the lateral ventricle. Interestingly, these authors have interpreted the presence of small βIII-tubulin$^+$ cells adjacent to a meshwork of GFAP$^+$/vimentin$^+$ glial cells in the SVZ of the lateral ventricle as evidence of putative neuroblasts (73).

Our findings in midgestational human fetuses mirror the previously described distribution of βIII-tubulin in the SVZ during the late stages (embryonic Day 18 to postnatal Day 5) of mouse telencephalic development (74) while further extending these observations by demonstrating coexpression of GFAP and βIII-tubulin in progenitor-like cells of the human SVZ. To date, most studies regarding the localization of βIII-tubulin in the VZ/SVZ in rodents focus either on phenotypic characterization of the intact SVZ in adult mice (18) or in the delineation of cell types derived from SVZ-isolated neurospheres of late embryonic or postnatal stages (53, 62). There is a widely held belief that βIII-tubulin expression in cells of the SVZ is proof for neuronal differentiation, an assumption that goes back to the immunohistochemical mapping study of Menezes and Luskin of βIII-tubulin in the developing mouse telencephalon (74). A major caveat in this regard, however, is that these authors had assumed from the outset that βIII-tubulin expression in cells of the SVZ is neuronal specific without having critically examined whether βIII-tubulin might also be expressed in glial progenitors and/or potential NSCs. To our knowledge, there are no studies to date offering a systematic appraisal of the ontology of βIII-tubulin relative to GFAP and nestin in the intact VZ/SVZ of the mouse telencephalon.

Preliminary studies from our laboratory have shown that βIII-tubulin is coexpressed with GFAP in the SVZ of the ganglionic eminence throughout the third trimester until early infancy (E. Dráberová, P. Dráber, and C.D. Katsetos, unpublished observations). At the same time, there is a progressive dissociation of βIII-tubulin and GFAP in the developing white matter and cerebral cortex of the human brain, where βIII-tubulin becomes neuronal, whereas GFAP is—for the most part—glial-restricted outside the SVZ (E. Dráberová, P. Dráber, and C.D. Katsetos, unpublished observations). Future studies are warranted to elucidate dynamic changes in the expression of GFAP and βIII-tubulin over time with resultant temporospatial segregation of these 2 proteins and loss of βIII-tubulin in quiescent astrocytes of the mature brain. In addition, it remains to be determined whether the differential expression of βIII-tubulin and GFAP in neurons and glia, respectively, corresponds to underlying functional developmental change(s).

Differential Staining Intensities Rendered by Different Monoclonal Antibodies to βIII-Tubulin

A noteworthy observation derived from the present study relates to the different staining intensities, with monoclonal antibodies recognizing different epitopes in the carboxy-terminal region of βIII-tubulin. Although all 3 anti-βIII-tubulin antibodies stained microtubule arrays, labeling with monoclonal antibody TuJ1 was more robust and generalized as compared with TU-20 and SDL.3D10. These distinct staining patterns most probably reflect different epitope location on microtubules recognized by each one of the antibodies used. The TuJ1 antibody was raised against chicken brain tubulin, and its epitope is located in the peptide covering the last 14 amino acids on the C-terminal end of chicken βIII-tubulin (75). Antibodies TU-20 and SDL.3D10 were raised against identical 8-amino-acid peptide from C-terminal end of human βIII-tubulin. In this region, the chicken and human βIII-tubulin differ at 2 amino acid residues (76). The epitopes recognized by antibodies TU-20 and SDL.3D10 can be masked owing to conformational changes and/or posttranslational 22modification(s) associated with this C-terminus region of βIII-tubulin.

Implications for Phenotypic Identity

The unexpected localization of βIII-tubulin in human fetal astrocytes has major practical and theoretical implications in stem cell research because it casts reasonable doubt about the assumed phenotypic identity of βIII-tubulin$^+$ cells in vitro as presumptive neurons (4, 5, 27). These findings attest to a constitutive expression of βIII-tubulin in fetal astrocytes and raise questions as to the assumed neuronal specificity of this protein in vitro and potentially in vivo. In addition, the findings help to understand the previously described coexpression of GFAP and βIII-tubulin in malignant gliomas (28, 30), probably reflecting a close phenotypic relationship of the latter to NSCs or partially committed glial progenitors.

The existing body of scientific literature contains a plethora of publications in the fields of adult neurogenesis (23) and stem cell transplantation research in which immunolabeling for βIII-tubulin is construed as evidence of a cell's presumptive neuronal identity or differentiation (9, 11–16). Previous studies have shown, however, that βIII-tubulin is not unique to neuronal cells (4, 5). We and others have previously reported that βIII-tubulin has been detected using the same monoclonal antibodies as in the present study in a variety of human cells and tissues such as Kulchitsky epithelial cells of the airway epithelium in the fetal lung (39), human spermatozoa (77), and follicular lymphoid cells (78).

Our findings have potential implications in the phenotypic characterization of developing brain cells by putting into question the assumed neuronal identity of βIII-tubulin-expressing cells in vitro and in situ. It remains to be elucidated whether the unexpected coexpression of βIII-tubulin along with GFAP and nestin signifies bipotential progenitor-like properties of fetal astrocytes or whether it denotes that βIII-tubulin is a constituent of microtubules in glial restricted precursor cells and their progeny during gliogenesis.

The coexpression of neuronal and glial antigens in astrocytes is of significant interest because of its potential relation to phenotype "plasticity." Are the barriers between the various neuroepithelial cell lineages less restrictive than is generally thought? Can neuronal or glial cells undergo "transdifferentiation" phenomena by changing their phenotype and antigenic profile? Alternatively, antigen coexpression can

simply mean that presumed phenotype markers are not specific enough to distinguish between different cell types during some, or all, developmental stages. The present study serves as a cautionary statement against using βIII-tubulin expression as proof of neuronal differentiation, as is often done in in vitro NSC experiments.

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