

Lineage Pathway of Human Brain Progenitor Cells Identified by JC Virus Susceptibility

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Multipotential human central nervous system progenitor cells, isolated from human fetal brain tissue by selective growth conditions, were cultured as undifferentiated, attached cell layers. Selective differentiation yielded highly purified populations of neurons or astrocytes. This report describes the novel use of this cell culture model to study cell type-specific recognition of a human neurotropic virus, JC virus. Infection by either JC virions or a plasmid encoding the JC genome demonstrated susceptibility in astrocytes and, to a lesser degree, progenitor cells, whereas neurons remained nonpermissive. JC virus susceptibility correlated with significantly higher expression of the NFI-X transcription factor in astrocytes than in neurons. Furthermore, transfection of an NFI-X expression vector into progenitor-derived neuronal cells before infection resulted in viral protein production. These results indicate that susceptibility to JC virus infection occurs at the molecular level and also suggest that differential recognition of the viral promoter sequences can predict lineage pathways of multipotential progenitor cells in the human central nervous system.

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The differentiation of central nervous system (CNS) stem and progenitor cells into neuronal and glial lineages is accompanied by the expression of specific intracellular molecules involved in the transcription of cell type-specific genes. The selective differentiation of human CNS progenitor cells into neural cell types provides a unique model to study the molecular regulation of cellular phenotypes as well as neurotropic viruses that target specific subpopulations of CNS cells. For example, the human polyomavirus, JC virus (JCV), demonstrates a restricted cellular host range and tropism in the CNS, targeting glial but not neuronal cells.^{1,2} Lytic infection of oligodendrocytes results in the fatal demyelinating disease, progressive multifocal leukoencephalopathy.^{1,2} JCV infects astrocytes both *in vivo* and *in vitro* but does not infect neuronal cells,^{3–6} presenting a potential application of the CNS progenitor cell culture system for further study of JCV susceptibility.

JCV is unique among most viruses in that viral binding and entry do not predict susceptibility to infection.⁷ Therefore, it is hypothesized that the selective tropism of JCV is governed by molecular determinants, namely, nuclear transcription factors located within susceptible cells. The promoter-enhancer region of JCV contains multiple sites for the nuclear factor-1 (NFI)

family of transcription factors,^{8–10} which includes four members, NFI-A, NFI-B, NFI-C, and NFI-X^{11,12} (also known as NFI-D). NFI has been implicated in the transcriptional regulation of several CNS-specific cellular genes^{12–17} and viral replication,^{18–21} including that of JCV.^{22–24} Recognition of the JCV regulatory region by these molecular factors regulates viral genome replication, protein synthesis, and the initiation of viral multiplication. Therefore, individual NFI members may be critically involved in restricted glial cell tropism in the CNS.

The human CNS multipotential progenitor cells, described in this study, were used as a unique *in vitro* model to study the molecular regulation of JCV infection and to examine the potential role of NFI transcription factors in initiating viral multiplication in specific subpopulations of CNS cell types. The data presented in this article are the first to our knowledge demonstrating JCV infection of a population of human CNS progenitor cells. These experiments indicate that susceptibility to infection depends not on viral binding and entry, but on intracellular factors. Notably, overexpression of one of the NFI class members, NFI-X, in the neuronal cells initiated JCV susceptibility. These data substantiate the importance of NFI-X recognition in the transcriptional regulation of JCV susceptibility

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and in the distinction between glial and neuronal lineage pathways.

Materials and Methods

Isolation and Culture of Human Central Nervous System Progenitor Cells

Human CNS progenitor cells were isolated from the telencephalon of an 8-week gestation human fetal brain, obtained in accordance with NIH guidelines. After dissection and dissociation, the cells were cultured directly into serum-free Neurobasal medium (Invitrogen, Gaithersburg, MD), supplemented with N2 components (Invitrogen), Neural Survival Factor (Clonetics, Walkersville, MD), L-glutamine (2mM), penicillin/streptomycin (100IU/ml), gentamicin (50µg/ml), 0.5% bovine serum albumin (Sigma, St. Louis, MO), and growth factors, basic fibroblast growth factor (25ng/ml bFGF; Sigma), and epidermal growth factor (20ng/ml EGF; Sigma). All cells were grown as attached cultures on poly-D-lysine-coated plasticware at 37°C in 5% CO₂. After several passages of growth factor selection, an undifferentiated population of multipotential progenitor cells was isolated, as determined by immunocytochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) for phenotypic marker expression. To differentiate progenitors into neurons, we switched the growth factors from bFGF/EGF to brain-derived neurotrophic factor (10ng/ml; Sigma) and platelet-derived growth factor A/B (10ng/ml; Sigma). For astrocytic differentiation, progenitor cultures were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD). The differentiation process lasted 20 days.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.02% Triton. Cells were then incubated with rabbit polyclonal antiserum against human nestin²⁵ or JCV VP-1 protein and mouse monoclonal antibodies against MAP-2 (Calbiochem, San Diego, CA), JCV large T protein,²⁶ β-III tubulin (Covance, Richmond, CA), or glial fibrillary acidic protein (GFAP; Covance). Texas Red or fluorescein-conjugated anti-rabbit or anti-mouse secondary antibodies were used as appropriate (Jackson ImmunoResearch, West Grove, PA). For double immunostaining with monoclonal antibodies against large T-protein (IgG1) and β-III tubulin (IgG2a), isotype-specific secondary antibodies were used (Southern Biotechnology, Birmingham, AL). Bisbenzimidazole (5µg/ml) was used to label nuclei of all cells. Immunofluorescent-labeled cells were examined on a Zeiss (Thornwood, NY) axiovert inverted microscope with appropriate fluorescence filters. Immunopositive cells in four fields of approximately 60 to 100 cells were counted for each antibody combination, performed in duplicate in at least three independent experiments.

In Situ Hybridization

DNA-DNA in situ hybridization was performed as described.²⁷ Biotinylated JCV DNA probe was visualized by diaminobenzidine reaction with avidin-biotin complex

(ENZO Biochemical, New York, NY). Cells were counterstained with hematoxylin.

Hemagglutination Titer of JC Virus

Hemagglutination assay was performed as previously described.²⁸ Hemagglutination titers were expressed as the reciprocal of the final dilution resulting in hemagglutination of human type-O erythrocytes.

Transfections of Plasmids and Infection with JC Virus

Cells were transfected with 1µg of plasmid DNA using either 1µg Lipofectamine 2000 (Invitrogen) or 5µg DOTAP (Roche Molecular Biochemicals, Indianapolis, IN) for 1 × 10⁵ cells, according to the manufacturer's instructions. The transfected plasmid DNA encoded either the entire Mad-1 strain of JCV²⁹ (pM1Tc), mouse Nfi-a¹¹ (pCHAmNF1-A1.1; accession number U57633), mouse Nfi-b¹¹ (pCHAmNF1-B2; U57634), mouse Nfi-c¹¹ (pCHAmNF1-C2; U57635), mouse Nfi-x¹¹ (pCHAmNF1-X2; U57636), human NF1-X²⁴ (pAT1, U18759), or the enhanced green fluorescent protein (Clontech, Palo Alto, CA) as a control. For infection experiments, 1 × 10⁵ cells were exposed to 333 hemagglutination units of the Mad-4 strain of JCV. Twenty-four hours after viral exposure, all medium was replaced with new medium. Immunocytochemistry and Northern blots were performed on cells 5 to 14 days after transfection and infection to assay for viral gene or protein expression.

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from cells using the RNeasy total RNA isolation system (Qiagen, Valencia, CA) followed by DNase I (10U/µg RNA; Boehringer Mannheim, Indianapolis, IN) treatment for 60 minutes. First-strand cDNA synthesis was performed on 1µg of total RNA using oligo dT primers and Superscript II reverse transcriptase (Invitrogen). cDNA product (1µl) served as template for specific PCR amplification of each mouse NFI transcription factor family member. A 5' PCR primer within the hemagglutination tag of all plasmids (5'-CCTTACGATGTACCAGACTATGC-3') was used along with selective mouse NFI class-specific primers: NFI-A (5'-GTCAGCTTCACTTGGCTGGC); NFI-B (5'-TGGAGAAGACAGCGACCTCTGCAG-3'); NFI-C (5'-CATCTCGCTCACGCACGAAG-3'); and NFI-X (5'-CCGCATCTCCTTGCTGGTTT-3'). PCR amplification consisted of 30 cycles using the following cycling conditions: 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C using the Perkin-Elmer 2400 thermal cycler. PCR products (30µl) were electrophoresed through a 1% agarose gel, stained in ethidium bromide, and photographed.

Generating Probes and Northern Blot Analysis

Class-specific RNA probes for NFI-A, -B, -C, -X and GAPDH, and DNA probes for JCV large T protein were generated for Northern blot analysis. Reverse transcription was performed as described above, followed by PCR with the following primers: NFI-A: (5'-GCCAGCCAAGTGAAGCTGAC-3'), (5'-GGTGTGCGAGTAGGTGGTGA-3'); NFI-B: (5'-ACCCAGCCAGCCATACATG-3'), (5'-TGTGTGCGACTCCAGGTATTCC-3'); NFI-C: (5'-TACTTCGT-

CGTGAGCGAGATG-3'), (5'-ACGAGATGCCTCCTTCC-ATGTC-3'); NFI-X: (5'-AAACCAGCAAGGAGATGCGG-3'), (5'-AGAACACGTCATCAACAGGGGTC-3'); JCV T: (5'-GAATAGGCAGGAATCCATGG-3') and (5'-GGAATGCATGCAGATCTACAGG-3'); GAPDH: (5'-GAGCTTGACAAAGTGGTCGT-3'); and (5'-CCACAGTCCATGCCATCACT-3').

PCR products were cloned using the TOPO-TA cloning system into pCR2.1 vector (Invitrogen). RNA probes were generated by linearizing plasmids with *Hind*III, synthesizing the antisense RNA strand from the T7 promoter, and incorporating ³²P-CTP. DNA probe for JCV T was generated by ³²P labeling of the purified JCV T PCR product.

For Northern blots, total RNA was extracted using RNeasy kit (Qiagen), with 15 μg electrophoresed through a 1% agarose-formaldehyde gel using the NorthernMax kit (Ambion, Austin, TX) and transferred to nylon membrane. Hybridization of ³²P-labeled probes was performed at the following temperatures: NFI-A, -B, and -C at 75°C; NFI-X at 65°C; JCV T protein at 52°C, followed by autoradiography on Kodak BioMax-MS film (Rochester, NY). The blots were rehybridized with the GAPDH probe (75°C) as a control. Densitometric analysis was performed to quantify the relative NF1 expression as compared with GAPDH using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Electromobility Shift Assay

Nuclear extracts (10 μg) were analyzed by competitive electromobility shift assay (EMSA), essentially as described.²⁷ Double-stranded NFI consensus oligonucleotides (5'-ATGGCTGCCAGCCAAG-3') labeled with γ-³²P-ATP were used as specific probes. Unlabeled double-stranded homologous or mutant NFI oligonucleotides (5'-ATTACTGCCAGCTGAG-3') were used as competitors (250-fold molar excess). The nuclear extracts/probe mixtures were electrophoresed through a 6% polyacrylamide-Tris-glycine gel, dried, and visualized by autoradiography, with Kodak BioMax-MS film. Densitometry was performed, as described above, to quantify the relative levels of NFI protein binding.

Results

Multipotential Progenitor Cell Isolation, Growth, and Differentiation

A renewable line of human multipotential CNS progenitor cells was utilized to examine the molecular regulation of JCV infection (Fig 1a). Immunocytochemistry demonstrates that 98% of the progenitor cells express the neuroepithelial stem cell marker nestin and incorporate bromodeoxyuridine, indicating active proliferation (see Fig 1b). These progenitor cells do not express markers for astrocytes, oligodendrocytes, microglia, or fibroblasts and have been maintained in an undifferentiated state for 25 passages or in excess of 1 year in culture, with no apparent change in cellular phenotype (data not shown). After neuronal differentiation of the multipotential progenitor cells, 50 to 70% of the cells expressed neuronal phenotypic markers β-III tubulin (see Fig 1c) or MAP-2 (data not shown).

This was accompanied by a halt in proliferation and reduced nestin expression (data not shown). Astrocytic differentiation of progenitors results in almost 100% expression of the astrocytic marker GFAP (see Fig 1d), with a proliferation rate similar to primary human fetal brain astrocytes. Neither the progenitor-derived neurons nor astrocytes expressed markers for oligodendrocytes, fibroblasts, microglia, or endothelial cells (data not shown). Several growth factor combinations previously shown to produce rodent oligodendrocytes were tested, but very few human oligodendrocytes were ever detected in these cultures.

Initiation of Infection Using JC Virions

To examine infectability of the progenitors and progenitor-derived neurons and astrocytes, we assayed cells for susceptibility to JCV infection as demonstrated by early viral protein (large T) expression, the replication of viral DNA, and late viral protein (VP-1) expression. Ten days after exposure to purified JCV, 5 to 7% of the progenitor cells showed signs of infection (Fig 2a–c), indicating JCV susceptibility. Progenitor-derived astrocytes were highly susceptible to JCV, with approximately 50% of the cells demonstrating infection (see Fig 2g–i), whereas neuronal cells remained nonsusceptible to infection (see Fig 2d–f). There was no observed toxicity in neuronal cells exposed to JCV (data not shown). Hemagglutination assays demonstrated higher virion production in progenitor-derived astrocytes than progenitor cells, and no detectable virions from progenitor-derived neuronal cells (Fig 3).

Transfection of JC Virus Genome

To specifically determine the ability of progenitors, neurons, and astrocytes to recognize the JCV promoter sequences and produce viral proteins, we transfected cells with a plasmid, pM1Tc, encoding the entire infectious JCV DNA genome. These experiments delivered the viral genome directly into the cells by liposome transfection, thereby bypassing the usual viral binding and entry mechanism. Ten days after transfection, JCV large T protein was detected in 2% of the progenitor cell population (Fig 4a, b) and 10% of the progenitor-derived astrocytes (see Fig 4f, g), but not in the progenitor-derived neuronal cells (see Fig 4c, d). This trend matched the results after exposure to infectious JCV particles, indicating that gene expression, and therefore viral susceptibility, is predominantly dependent on intracellular factors. Control enhanced green fluorescent protein transfections determined approximately equal transfection efficiency in progenitor cells (6%) and progenitor-derived neuronal cells (7%) but higher efficiency in progenitor-derived astrocytes (18%) (data not shown).

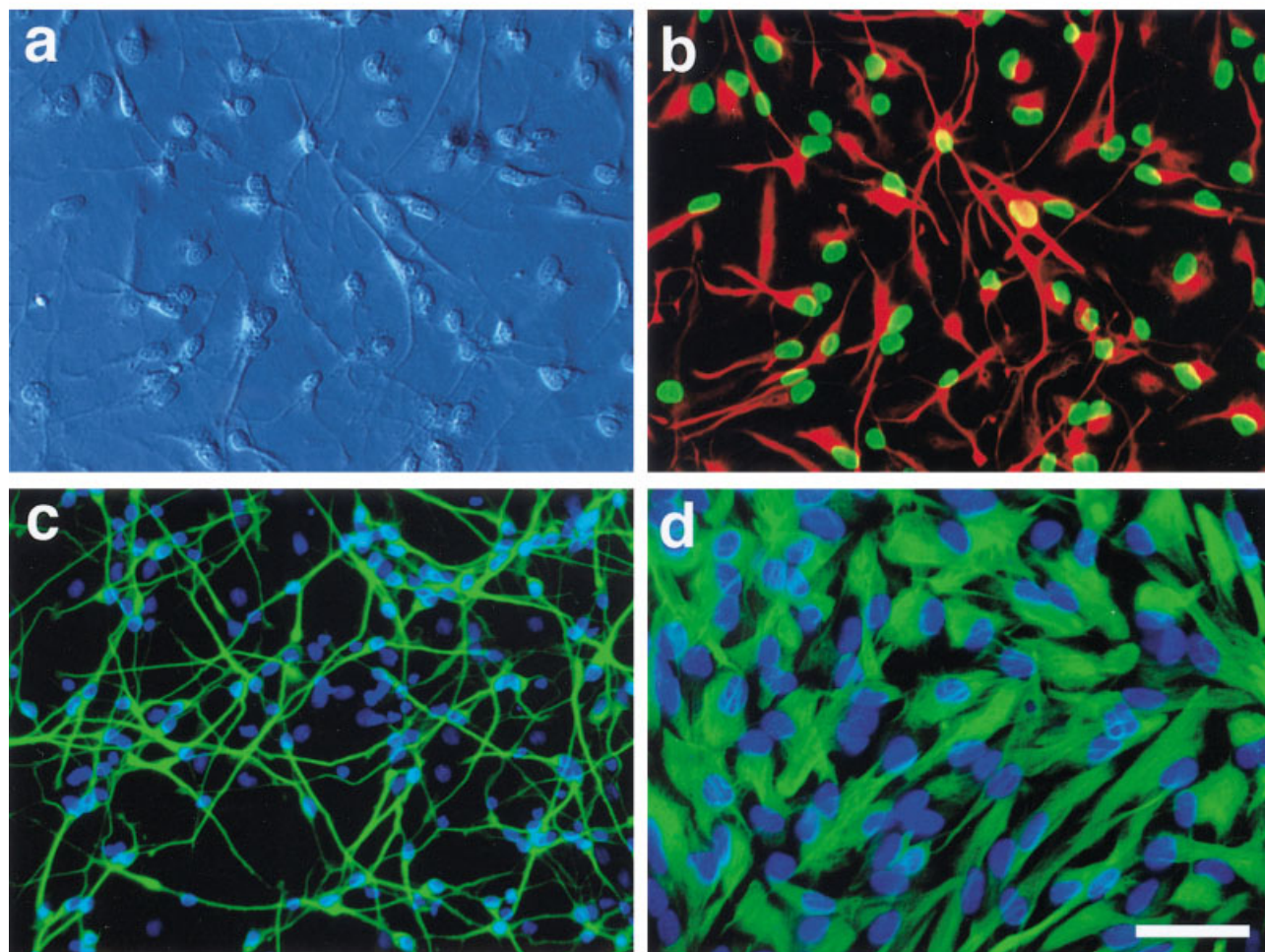


Fig 1. Immunofluorescence staining of the human central nervous system progenitor cells. (a) Hoffman optic photomicrograph of the undifferentiated multipotential progenitor cells. (b) Immunofluorescence staining of progenitor cells in panels a, incorporating bromodeoxyuridine (green) and staining with nestin (red). (c) Progenitor cells differentiated into neurons and immunostained with antibodies against β -III tubulin (green), with nuclei stained with bisbenzimidide (blue). (d) Progenitor cells differentiated into astrocytes and immunostained with antibodies against GFAP (green), with nuclei stained with bisbenzimidide (blue). Scale bar in d = 50 μ m.

Molecular Regulation of JC Virus Infection

Previous studies have suggested that the NFI family of transcription factors may play a role in regulating JCV genome replication.^{8–10,24} Therefore, we examined functional binding of nuclear proteins, isolated from the progenitor cell culture system, to NFI DNA sequences by competitive EMSA. Although progenitor cells, neurons, and astrocytes all contained NFI proteins that bound to the consensus NFI sequence, the relative amount of binding was less in nonpermissive neuronal cells, as compared with the JCV susceptible astrocytes or progenitors (Fig 5). To further assess the expression levels of each NFI family member, we performed Northern blot analysis using newly developed NFI class-specific RNA probes (see Materials and Methods). Quantification by densitometric analysis showed that progenitors, as well as progenitor-derived

neurons and astrocytes, expressed similar levels of NFI-A and NFI-C (Fig 6). However, the neuronal cells expressed approximately 2-fold more NFI-B than progenitors and 10-fold more than astrocytes. Furthermore, the highly susceptible progenitor-derived astrocytes expressed twofold more NFI-X than the nonpermissive progenitor-derived neuronal cells (see Fig 6).

We conducted further studies to determine the functional significance of NFI family members in the transcriptional regulation of JCV susceptibility. Expression plasmids encoding each of the NFI transcription family members were individually transfected into parallel neuronal cell cultures 24 hours before infection with JCV (Fig 7a). RT-PCR demonstrated the specific expression of each transfected NFI class member, distinguishable from endogenously expressed NFIs (see Fig

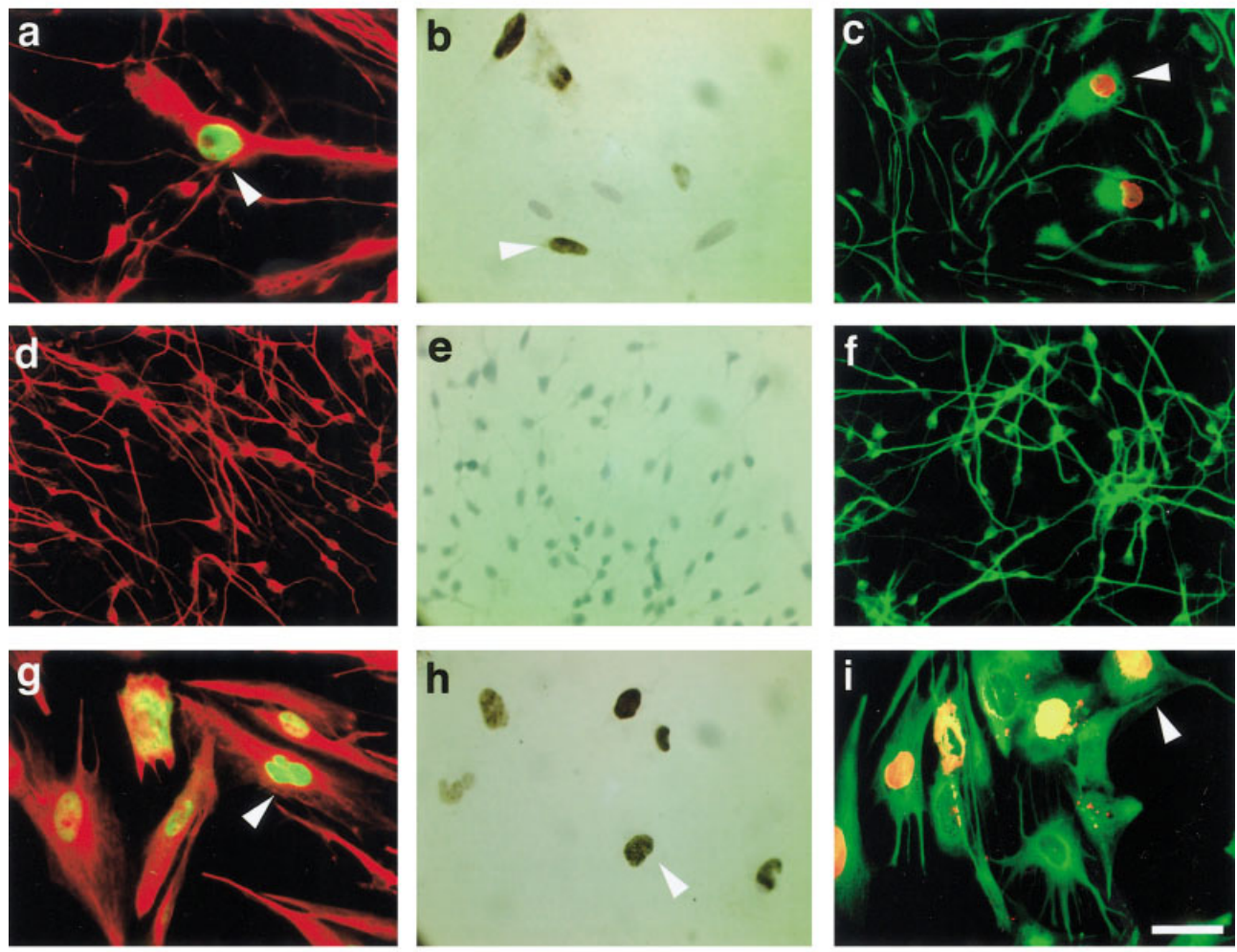


Fig 2. Viral genome and protein detection after JC virus (JCV) exposure. (a–c) Progenitor cells; (d–f) progenitor-derived neuronal cells; (g–i) progenitor-derived astrocytes after exposure to JCV. (a) Progenitor cells immunostained for nestin (red) and for viral large T protein (green). (b) In situ hybridization to detect replicated viral DNA (brown). (c) Immunostained cells for nestin (green) and viral late protein VP-1 (red). (d) Progenitor-derived neuronal cells immunostained for β -III tubulin (red) and for viral large T protein (green). (e) In situ hybridization to detect replicated viral DNA (brown) was negative. (f) Immunostained cells for β -III tubulin (green) and viral late protein VP-1 (red), which was negative. (g) Progenitor-derived astrocytes immunostained for GFAP (red) and for viral large T protein (green). (h) In situ hybridization to detect replicated viral DNA (brown). (i) Immunostained cells for GFAP (green) and viral late protein VP-1 (red). Scale bar in i = 25 μ m. Arrowheads indicate viral antigen-positive cells.

7a). We observed that transfection of NFI-X into the progenitor-derived neuronal cells before JCV infection initiated JCV susceptibility, as indicated by early viral gene, large T expression (see Fig 7b). This was further supported by the immunocytochemical detection of viral T protein in the nuclei of β -III tubulin-positive neurons (see Fig 7c). Transfection of progenitor-derived neurons with NFI-A, NFI-B, or NFI-C had no effect on viral gene expression or viral protein synthesis (see Fig 7b). To examine a potential negative regulatory role of NFI-B in JC viral susceptibility, we also transfected NFI-B into the astrocyte population, followed by exposure to JC virus. The results from these experiments demonstrated no decrease in viral T pro-

tein expression, as measured by RT-PCR or Northern blot (results not shown).

Discussion

The human CNS-derived, multipotential progenitor cell culture system was established from human fetal brain tissue by means of selective growth isolation and differentiation. These progenitor cells initially were cultured as stable, attached cell layers, in contrast with other reports in which cells were grown only as spheres in suspension.^{30–32} In accordance with previous publications, both EGF and bFGF were required for maintenance of the cells in an actively proliferating, undifferentiated phenotype.^{30–32} However, the progenitor

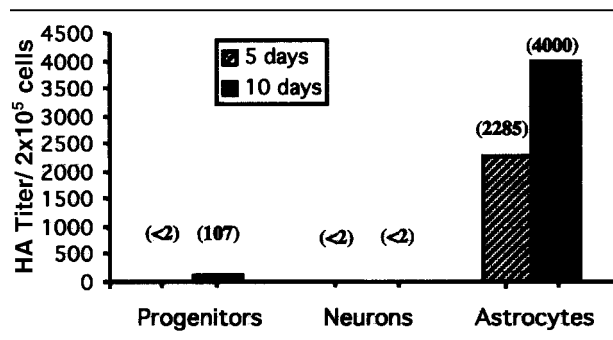


Fig 3. JCV virion production after JCV exposure. Progenitor cells, progenitor-derived neurons, and astrocytes were exposed to JCV for 24 hours and assayed for virion production by hemagglutination (HA) titer assay. HA titer obtained from cells 5 and 10 days after exposure to JCV.

cells established in this study are unique in that they can remain undifferentiated either as attached layers or as floating neurospheres, as verified by RT-PCR and immunocytochemistry (see Fig 1 and C. Messam, J. Hou, and E. Major, unpublished observations). The ability to grow progenitor cells as attached cultures facilitates and greatly extends the application of these cells for experimental purposes.

Highly purified populations of neurons and astrocytes, demonstrated by the expression of cell type-specific phenotypic markers, were selectively generated from the progenitor cells, depending on cell culture conditions (see Fig 1). A stable and virtually unlimited source of homogeneous multipotential progenitor cells has significant utility in examining not only cellular differentiation, but also the selective expression of exogenous genes resulting from viral infection. Furthermore, the culture system subsequently could be used for therapeutic transplantation in animal models of neurological disorders. As yet, it has not been possible to differentiate the progenitor cells from this study into oligodendrocytes. Although oligodendrocytes have been derived from rodent CNS stem cells,^{33,34} there has been sparse evidence for the isolation of purified human oligodendrocytes from either human fetal brain or from other human CNS stem/progenitor cells.^{35,36}

The data reported here describe the three purified populations of CNS cells and their different molecular responses to infection by the human polyomavirus, JCV. JCV demonstrates a highly restricted cellular host range, with productive infection in oligodendrocytes and astrocytes, but not in neuronal cells.^{1,2} This study confirmed previous observations that human cultured astrocytes are highly susceptible to JCV infection,^{24,27} demonstrated by efficient viral replication and virion production as a result of either JCV virion exposure (see Fig 2g-i and 3) or transfection of infectious JCV DNA (see Fig 4). In contrast with the astrocytes, the data also confirmed that neuronal cells were nonpermissive

to JCV infection, demonstrating no signs of infection by either direct JCV virion exposure (see Fig 2d-f and 3) or transfection of DNA directly into the cells (see Fig 4). These data, along with the expression of cell type-specific phenotypic markers, could be used to predict the lineage pathways of the progenitor-derived cell types.

Interestingly, the progenitor cells also demonstrated viral replication and virion multiplication upon infection by virus as well as transfection of viral DNA. The susceptibility of human CNS progenitor cells to JCV infection is a novel finding of this study. Closer examination of brain tissue from progressive multifocal leukoencephalopathy patients will be necessary to determine if, in fact, in vivo stem and progenitor cells show evidence of viral DNA or virions. These studies are currently under way.

Susceptibility to JCV infection in the progenitor cell culture model was not dependent on receptor binding and entry. This was especially evident in the neuronal population, where the cells remained nonsusceptible to infection despite the fact that the viral genome was transfected directly into the cells (see Fig 4), bypassing the receptor binding and entry mechanism. These results contrast with the robust infection exhibited by the progenitor-derived astrocytes and, to a lesser degree, progenitor cells, in response to transfection (see Fig 4). Although no viral antigen was detected in neurons 3 weeks after JCV plasmid transfection, it is known to result in infectious virus production after 5 to 7 weeks in primary astrocytes.³⁷ A recent report demonstrated the cellular binding and entry of JCV in the absence of subsequent viral genome replication or viral protein synthesis.⁷ These data support the hypothesis that, unlike most viruses, susceptibility to JCV infection is determined at the molecular level and less at the level of binding and entry. Specifically, JCV susceptibility is conveyed by the recognition of the viral regulatory region by transcription factors within the nuclei of susceptible cell types.

The JCV viral promoter-enhancer sequences contain several binding sites for the NFI transcription factor family that may influence viral genome replication and transcription.^{8,10,23,24} Functional binding of the NFI proteins, extracted from each of the purified cell populations, to a consensus NFI binding sequence was assayed by EMSA. The results showed that NFI proteins from the nonsusceptible neuronal cells consistently showed a marked decrease in binding to the consensus sequence, as compared with the progenitor cells or the progenitor-derived astrocytes (see Fig 5). Furthermore, the different migration patterns of the gel-shifted bands suggested that each cell type possessed different combinations of the NFI class members. Northern blot analysis showed that the nonsusceptible progenitor-derived neurons expressed much higher levels (10-fold)

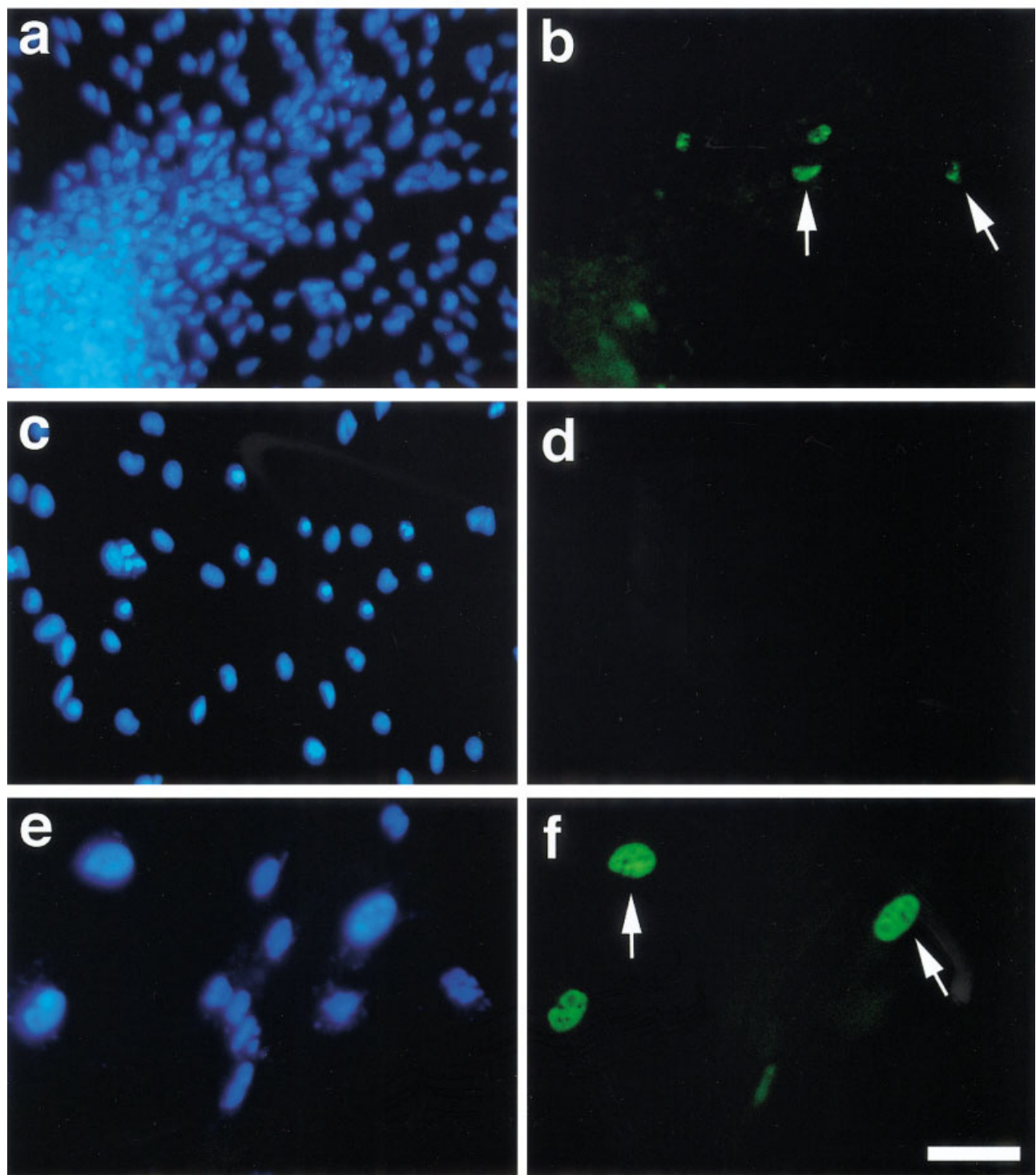


Fig 4. Immunocytochemistry for viral large T protein in cells transfected with the JCV DNA. Each horizontal row represents the same field of cells. Top panels (a, b) are progenitor cells, the middle panels (c, d) are neuronal cells, and the bottom panels (e, f) are glial cells. Panels a, c, and e are immunostained with nuclear dye bisbenzamide. Panels b, d, and f are stained with antibodies against JCV large T protein. Arrows indicate T-protein-positive cells. Scale bar in f = 25 μ m.

of NFI-B than the astrocytes, whereas the highly susceptible progenitor-derived astrocytes expressed twofold more NFI-X than neurons (see Fig 6). These data sug-

gest critical roles for NFI-X and/or NFI-B in JCV susceptibility and the expression of viral genes in the progenitor and progenitor-derived cells.

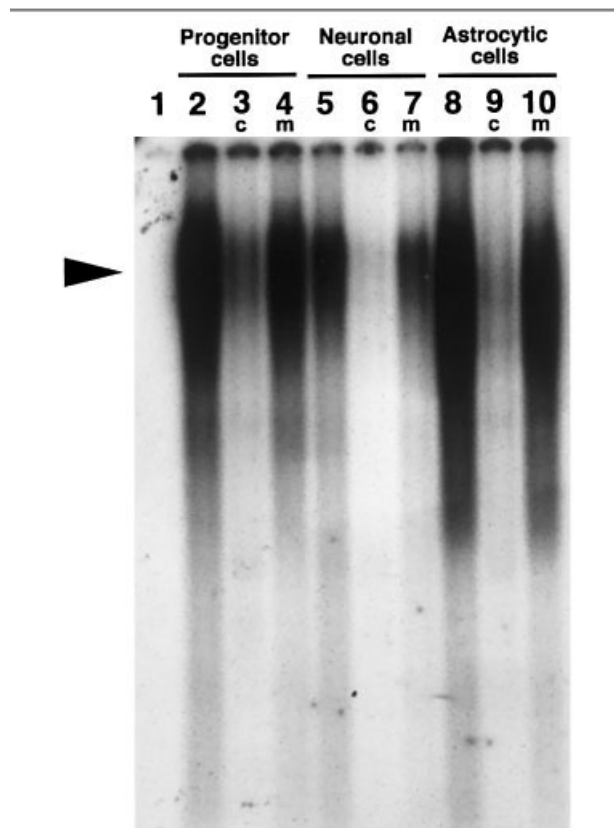


Fig 5. Competitive electromobility shift assay analysis of nuclear protein binding. Nuclear extracts from progenitor cells (lanes 2–4), progenitor-derived neuronal cells (lanes 5–7), and progenitor-derived astrocytic cells (lanes 8–10) and run with a radiolabeled oligonucleotide containing the NFI binding site. Competitors were either unlabeled cold homologous oligonucleotides (c) or unlabeled mutant oligonucleotides (m). Arrowhead indicates the position of the specific gel-shifted band, present in all cell nuclear extracts. Lane 1 contains the probe without added nuclear extract and shows migration off the gel.

To further investigate the functional role of each NFI class member in the regulation of JCV susceptibility, we independently transfected the nonpermissive neuronal cells with plasmids encoding NFI-A, NFI-B, NFI-C, or NFI-X and exposed to infectious virions (see Fig 7). Overexpression of NFI-X initiated JCV susceptibility in the previously nonsusceptible neuronal cells, as determined by large T expression and protein synthesis (see Fig 7b, c). However, overexpression of the other NFI classes (A, B, and C) had no effect, strongly implicating a specific regulatory role for NFI-X in initiating activation of JCV gene expression. A recent study in our laboratory similarly demonstrated initiation of JC viral gene expression in nonsusceptible, macrophage-like cells following transfection of a plasmid encoding NFI-X.²² Those data from the hematopoietic system, in addition to this study of human CNS cells, indicate that the NFI-X transcription factor

is important in the recognition of JC viral promoter sequences and the initiation of viral gene transcription. However, the levels of expression of NFI-X alone may not explain why neurons are not susceptible to JC infection, because progenitors are also susceptible and have a similar level of NFI-X expression as the neuronal cells. Although other factors may regulate different aspects of viral replication, NFI-X could be a molecular determinant of JCV susceptibility involved throughout viral pathogenesis.

To determine whether NFI-B plays a role in inhibition of JCV susceptibility, we performed experiments in which NFI-B was overexpressed in astrocytes before JCV infection. No decrease in T-protein gene expression as determined by Northern blot analysis and RT-PCR was observed (data not shown). It is possible that a higher percentage and/or level of expression of NFI-B in astrocytes may be required to see an effect. NFI-B also may inhibit JCV infection at a point other than at the level of T-protein expression. In addition, NFI-B may work in conjunction with other factors to inhibit JCV susceptibility. The role of other transcription factors involved in initiating JCV susceptibility and inhibiting infection is an active area of further research.

This is the first study, to our knowledge, to suggest that the expression and binding patterns of the NFI class members are different in neuronal and glial lineage cells. This corroborates previously published work indicating the importance of NFI transcription factors in CNS development.^{11,12,38} Progenitor-derived neuronal cells express all NFI family members (see Fig 6) but demonstrate low NFI protein binding (see Fig 5). This apparent discrepancy can be explained by several fac-

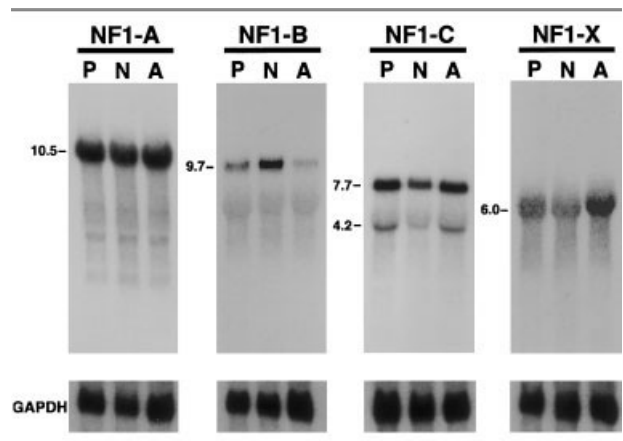


Fig 6. Northern blot analysis of NFI-A, -B, -C, and -X expression. Total RNA was extracted from progenitor cells (lanes P), neuronal cells (lanes N), and astrocytic cells (lanes A). ³²P-labeled NFI-A, -B, -C, and -X class-specific RNA probes were hybridized to each RNA blot. The marker on the left of each blot indicates the molecular weight in kilobases for the mRNA species. Each blot was later reprobed with a radiolabeled RNA probe against GAPDH.

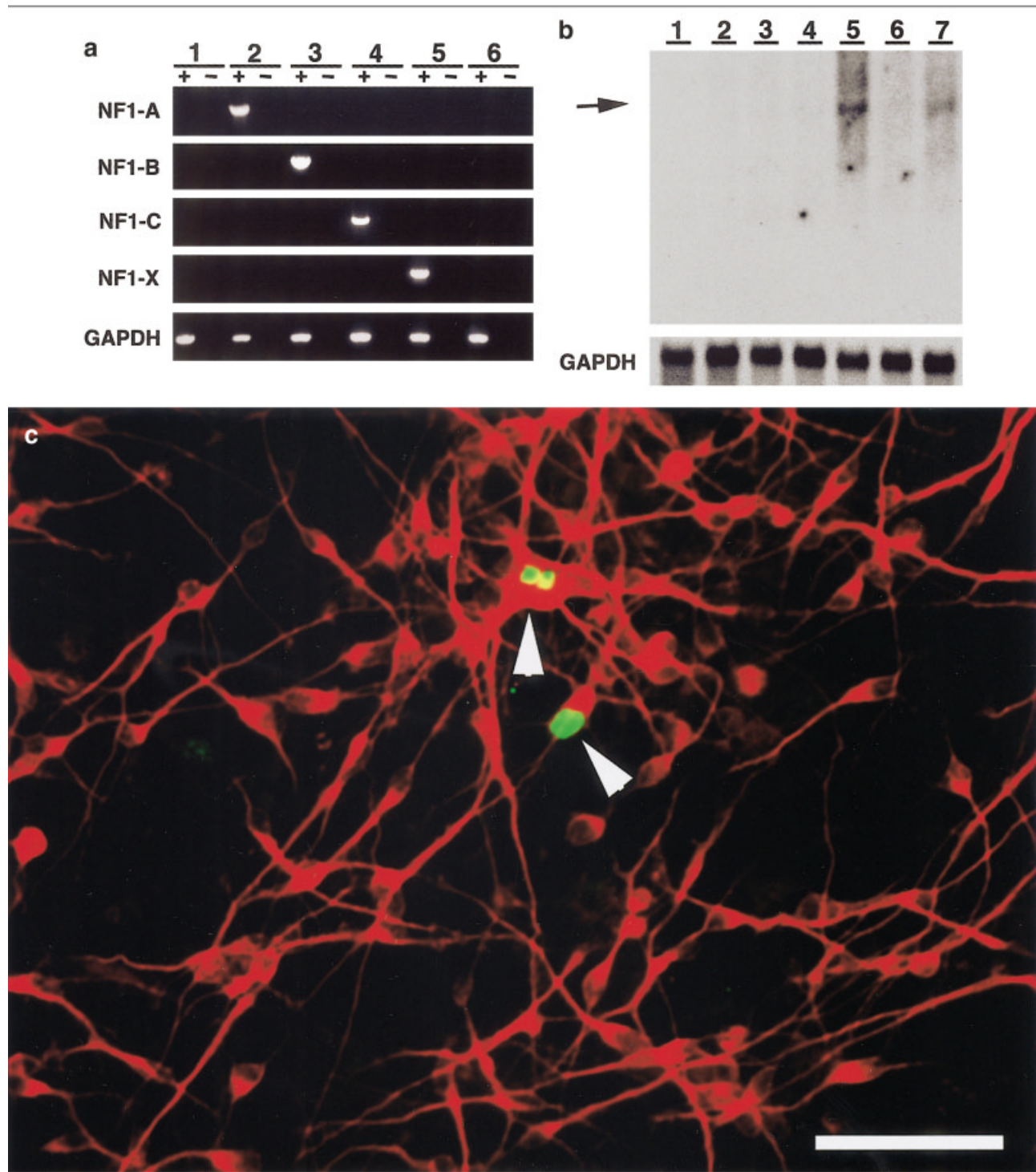


Fig 7. JCV susceptibility for progenitor-derived neurons overexpressing each NFI family member. (a) Reverse transcription polymerase chain reaction (RT-PCR) detection of the expression of each transfected NFI transcription factor in progenitor-derived neuronal cells using class-specific primers. Lane 1 and 6 were untransfected neuronal cell controls, whereas lanes 2 to 5 were transfected with plasmids encoding NFI-A (lane 2), NFI-B (lane 3), NFI-C (lane 4), and NFI-X (lane 5); lane 1 was uninfected and lanes 2 to 6 were infected with JCV. RT-PCR was performed with or without reverse transcriptase (plus and minus signs). (b) Northern blot analysis of mRNA expression of JCV T. Lanes 1 to 6 are total RNA with the same conditions as in a. Lane 7 contains RNA from progenitor cells infected with JCV. The blot was probed with a specific radiolabeled probe against JCV T RNA. The arrow on the left indicates the expressed mRNA species for JCV T gene. The blot was later reprobed for GAPDH. (c) Immunocytochemistry for JCV T protein in neuronal cells transfected with NFI-X and infected with JCV. Cells were immunostained with antibodies against β -III tubulin (red) and JCV = protein (green). Arrowheads indicate neuronal cells expressing viral protein. Scale bar represents 50 μ m.

tors. Because NFI proteins form homodimers or heterodimers to bind to the promoter-enhancer region of cellular or viral genes, there may be competition for NFI binding sites between various NFI protein subclasses and alternative splice variants^{39–42} as well as competition from adjacent or nearby transcription-binding factors which may also influence binding capability. Alternatively, NFI DNA-binding activity may also be sensitive to oxidative states.^{43,44} Future studies could be directed at determining the interactions of transcription factors in the JC viral promoter-enhancer region.

Preliminary studies have been performed that further suggest the lineage-specific recognition of viral sequences. Infection of progenitors followed by differentiation into astrocytes results in increased viral genome replication and protein expression. However, infection of progenitors followed by differentiation into neurons decreases viral replication and protein production. Therefore, the differential transcriptional recognition of JC viral promoter sequences observed using the progenitor cell culture model can distinguish between cells of glial and neuronal lineage. Furthermore, the progenitor cell culture model could have significant utility in examining other molecular differences existing between cells of different lineages during and after differentiation.

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