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Characterization of a brain tumor cell line established from transgenic mice expressing the vasopressin SV-40 T antigen

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Abbreviations: FISH, fluoresent *in situ* hybridization; SV, simian virus; VP, vasopressin

Abstract

We previously reported that transgenic mice produced with a transgene consisting of the SV40 T antigen and vasopressin without the 3'-flanking region exhibit brain tumors and lymphoma. In this study, transgenic mice were produced with the fusion gene containing the SV40 T antigen and the whole vasopressin gene with the 3'-flanking region. Six transgenic mice were generated, five which died after 2-6 weeks. The remaining founder mouse was investigated for fusion gene expression and tumor progression at the age of 6 weeks. Brain tumor cells were characterized for phenotypes and transgene expression. During *in vitro* cell cultures, the phenotypic appearances at 10, 20, and 30 passages were as a uniform monolayer with similar growth rates. The site of SV40 T antigen integration was in the A2 region of chromosome 11, and SV40 T antigen was expressed at the same level in cells of both earlier and later passages. Thirty passages were probably insufficient to reach crisis and immortalization. These cells enriched brain tumor cell compositions with astrocytes and neuronal cells.

Keywords: astrocyte cell; brain tumor cell; neuronal cell; transgenic mice; vasopressin

Introduction

We previously reported the generation of transgenic mice *via* microinjection of the fusion gene (pVPSV.IGR2.1) consisting of the SV40 T antigen and mouse vasopressin (VP) without the 3'-flanking region (Lee *et al.*, 2003). Of these mice, 21% developed brain tumors at 5 weeks and the remaining mice developed brain tumors after 24 weeks. Histological analysis of the transgenic mice revealed primitive neuroectodermal tumors (PNET) in the brain with lymphoma in the spleen and lymph nodes.

The PNETs of the central nervous system are a family of brain tumors and embryonic malignant tumors that account for 20% of all midbrain tumors in children. These tumors are characterized by primitive histological features resembling neuroepithelial stem cells from the embryonic neural tube or immature progeny (Lee *et al.*, 2003). pVPSV.IGR2.1 transgenic mice provide a valuable mouse model system for investigating PNET tumorigenesis in the brain and lymphoma in the lymph nodes and spleen. However pVPSV.IGR2.1 transgenic mice died after 24 weeks with tumors in the brain so this model is not useful for study of tumor progression.

The regulatory regions for VP gene expression are still unclear and the IGR domains contain various transcription binding sites that control tissue-specific expression and may have functions that have yet to be elucidated (Ratty *et al.*, 1996; Jeong *et al.*, 2001). SV40 is a DNA tumor virus that produces oncogenicity *in vivo* by inducing brain tumors in rodents (Tognon *et al.*, 1996). Although the SV40 T antigen and derived cell lines exhibit clonal nonrandom chromosomal abnormalities, little is known about the event that causes chromosomal instability and rearrangement (Tognon *et al.*, 1996). In order to study tumorigenesis that occurs in the

central nervous system, a cell line that resembles astrocytes and neuronal cells is required.

We established a brain tumor cell line from transgenic mice for tumor genetics and physiology studies. This is a novel model system in which the molecular mechanism of cellular differentiation associated with the central nervous system can be analyzed. We generated pVPSV.IGR2.1 transgenic mice and established a cell line that was characterized by phenotype, growth rate, and expression of astrocytes and neuronal markers.

Materials and Methods

Preparation of the VP transgene

The transgene plasmid pVPSV.IGR3.6 was initially constructed by replacing the chloramphenicol acetyl transferase (CAT) reporter gene with the SV40 T antigen gene (Jeong *et al.*, 2001). The transgene in the pSE280 vector (Invitrogen, Carlsbad, CA) contains the 3.6 kb IGR section possessing the mouse VP gene of the 5'- and 3'-flanking regions, including all exons and introns. The SV40 T antigen gene (ACTT, Manassas, VA) was fused at exon III (Figure 1). The SV40 T antigen gene was prepared by PCR amplification and *Not* I digestion of the PCR product, which was inserted into the *Not* I site of the transgene plasmid. The primers used were 5'- ATA TAG CGG CCG CGC AAA GAT GGA TAA AGT TTT AAA CAG -3' and 5'- ATT ATG CGG CCG CAT TCA TTT TAT GTT TCA GGT TC -3'.

Production and identification of transgenic mice

Linear transgene DNA fragments containing the *Bam* HI/*Eco*47 III fragment that was excised from the pVPSV.IGR3.6 plasmid were purified in a 10-40% sucrose gradient, then dialyzed against 10 mM Tris-Cl (pH 7.4) and 0.1 mM EDTA. Transgenic mice (BCF1: C57BL/6 × C3H hybrid) were produced using microinjection of transgenes at a concentration of 3 μg according to standard protocols (Ryoo *et al.*, 2001; Lee *et al.*, 2003). Following microinjection, viable conceptuses were transferred to ampullae of pseudopregnant ICR mice. The presence of the transgene was determined in tail genomic DNA through PCR and southern blot analysis.

Establishment of a cell line

Primary cell cultures from midbrain tumors of one of the transgenic mice were prepared as a source of brain tumor cells. Tissue sections of midbrain tumors were minced and dispersed at 37°C for 30 min in Hanks Balanced Salt solution containing 10 mg/ml collagenase (Cooper Biomedical, Malvern, PA) and 10 μg/ml DNAse I (Sigma, St. Louis, MO). The cells were washed in PBS and subsequently plated in Dulbecco's Modified Eantigenle Medium (Gibco Laboratories, Grand Island, NY) that consisted of 10% fetal calf serum (Hycolon Laboratories, Lugan, UT), 4.5 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO). Floating and weakly adherent cells were transferred to fresh plates every 3 days.

SV40 T antigen determined by FISH

Cultured cells after 30 passages were fixed on silan-coated slides (Fisher Scientific, Pittsburgh, PA) and processed for FISH (Fluorescent In Situ Hybridization) using a digoxigenin-labeled SV40 T antigen DNA probe and a chromosome *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ) on an automated *in situ* hybridization instrument (Ventana Gen II, Ventana Medical Systems). The amount of probe hybridization mixture that was used was calculated relative to the target area (10 ml of probe mixture per 484 mm^2) of fixed cell area). Denaturation was performed at 69°C for 5 min before slides were incubated overnight at 37°C with the hybridization probe. After overnight hybridization and three post-hybridization stringency washes, fluorescence as a labeled antidigoxigenin detection reagent was manually applied to the slide sections at

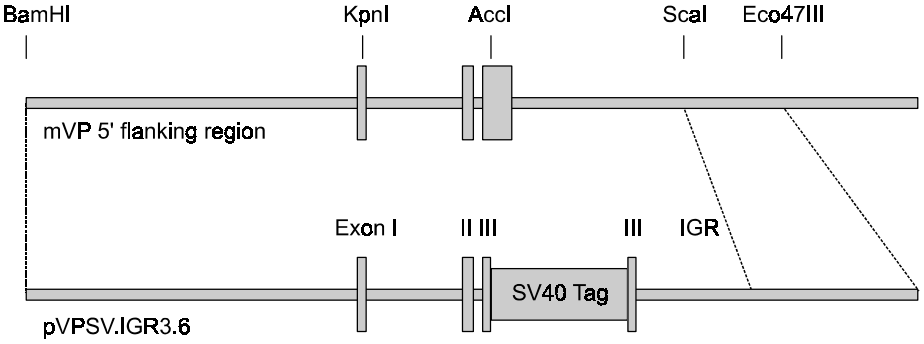

Figure 1. Vasopressin (VP)-transgene constructs. The transgenes contain 3.6 kb of the IGR consisting of the 5'- and 3'-flanking regions, and also contain all exons and introns of the mouse VP gene. The SV40 T antigen (Tag) was fused at the end of exon III.

37°C for 28 min. After removing the slides from the instrument, each slide was counterstained with 18 ml of propidium iodide antifade (1 : 2). Copy numbers for the SV40 T antigen gene were evaluated microscopically (Zeiss Axioskop 50 fluorescence microscope, Carl Zeiss, Thornwood, NY) at a magnification of × 100. Amplification scoring was based on fluorescence. The probe displayed a single fluorescent spot for each SV40 T antigen gene copy. The expected number of SV40 T antigen spots per normal or un-amplified tumor cell was two, or four in dividing cells. A minimum of 100 cells were evaluated for each specimen. Tumors were considered to be amplified for the SV40 T antigen gene when at least 20 cells displayed five or more spots per cell.

Immunoperoxidase cell staining

Brain tumor cells were cultured on a 6-well plate in a humidified incubator under 5% CO_2 at 37°C. The cells were washed with PBS and then fixed in -10°C methanol for 5 min. Fixed cells were incubated for 5 - 10 min in PBS that contained 0.1 to 1% hydrogen peroxide to quench the endogenous peroxidase activity. Immunoperoxidase staining required fixed cells to be incubated in a mixture of 1.5% normal blocking serum in PBS for one hour, followed by incubation with the primary antibody at room temperature for 30 min. After washing with three changes of PBS, each for 5 min, filters the cells were incubated for 30 min with a biotin conjugated secondary antibody, washed extensively with PBS, then incubated with an avidin biotin enzyme reagent (ABC Staining System, Santa Cruz Biotechnology, Inc., CA).

Western blot analysis

Homogenized cells were used to determine SV40 T antigen expression in the brain tumor cells. The

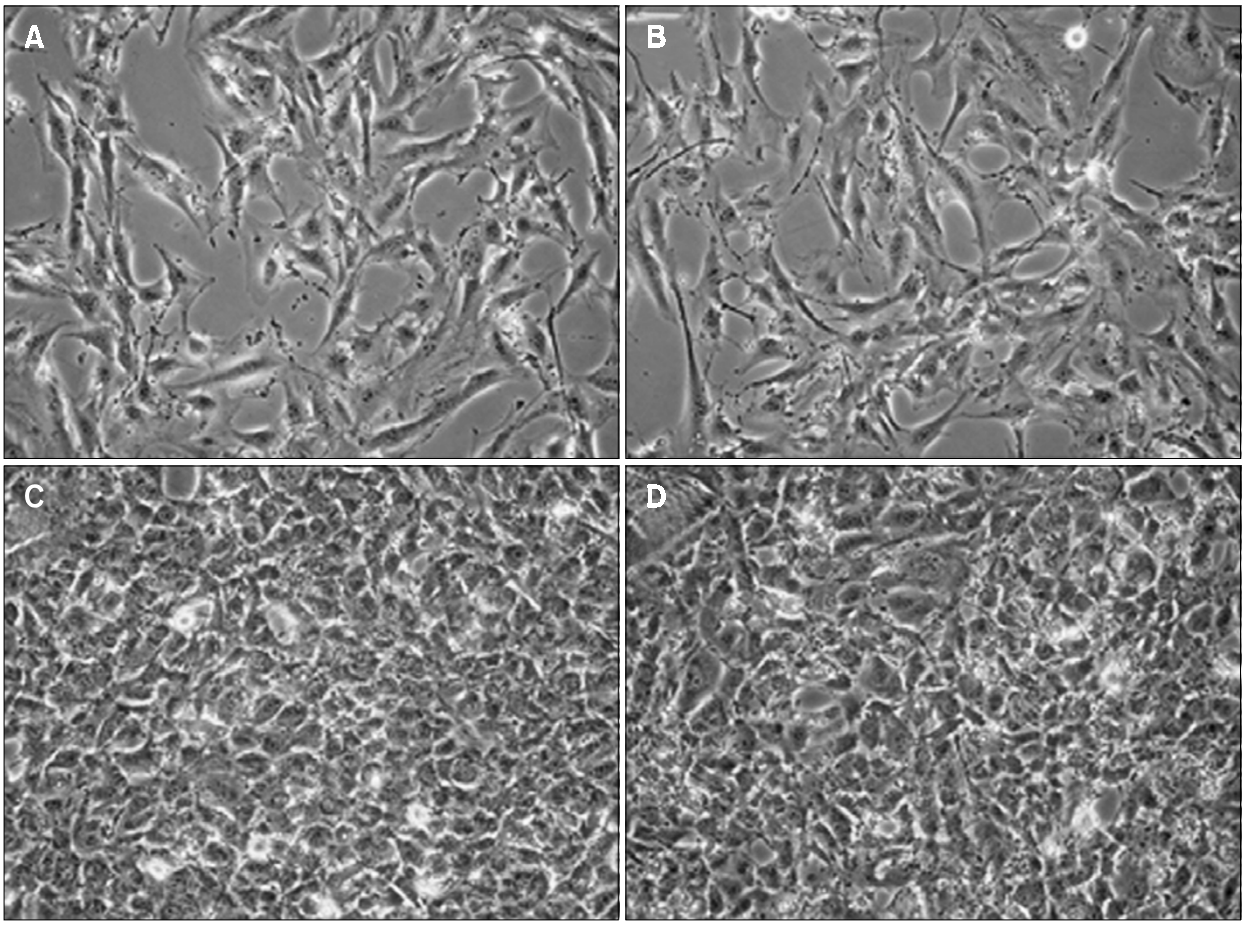

Figure 2. Primary cell cultures from a midbrain tumor of a pVPSV.IGR3.6 transgenic mouse. Brain tumor cells (2×10^4 cell/well) in DMEM supplemented with 10% FBS were seeded in plates and were incubated at 37°C for 3 days. At 10 passages (A, C) and 30 passages (B, D); 1 day culture (A, B), 3 day cultures (C, D). × 200

protein concentration levels were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. Equal amounts of protein from each cell homogenate were subjected to 8 % SDS-PAGE and then transferred to a nitrocellulose membrane filter. This filter was blocked in Tris-buffered saline containing 5 % BSA, 50 mM Tris-HCl of pH 7.5, 0.15 M NaCl, and 0.1% Tween-20 (TBST) at room temperature for 3 h. The filter was then washed with TBST and blotted with a monoclonal antibody against the SV40 T antigen (Pab 101, Santa Cruz Biotechnology, Inc.). The SV40 T antigen was localized using an ELC system (Amersham, Buckinghamshire, UK).

Results

Characterization of the brain tumor cell line derived from the midbrain tumor

Tumor tissues that were isolated from the surviving founder transgenic mouse were dispersed and maintained in culture for several months (Figure 2). This resulted in the generation of a novel cell line derived from the midbrain tumor of the pVPSV.IGR3.6 transgenic mouse. The balance between cell proliferation and cell death was maintained for several months (30 passages) without any sign of senescence. It has been reported that SV40 T transformed human bronchial epithelial cells that were cultured for 2 yrs were nontumorigenic, as assayed by *s.c.* injection of these cells into athymic nude mice (Lu *et al.*, 1998). No tumors resulted when SV40 T antigen gene transformed cells were injected into nude mice, which suggests that this cell line was still in a premalignant stage (data not shown). During the development of these features, the morphological appearance of the cells after 10, 20, and 30 passages was as a uniform monolayer with a similar growth rate (Figure 2 and 3).

SV40 T antigen determination by FISH

To determine the chromosomal location of the integrated SV40 T antigen gene, painting fluorescence *in situ* hybridization (FISH) analysis was performed. Cultured cells after 30 passages were used to produce chromosome spreads that were hybridized with the biotinylated SV40 T antigen probe. The integrated SV40 T antigen gene was localized to chromosome 11, A2 (Figure 4).

Immunoperoxidase cell staining and transgene expression

The coincidence that the SV40 T antigen transgenic line (at least pVPSV40.IGR3.6) exhibited the same degree of SV40 T antigen expression and genomic stability at all passages is remarkable. It is possible that only SV40 T antigen integration at chromosome 11, A2 is necessary for continuous expression of the SV40 T antigen protein and for survival of these cells. After 30 passages, the cells were stained with GFAP (90% positive cells), then NF150 antibody (10% positive cells) expression was determined (Figure 5). These midbrain tumor cells expressed the integrated SV40 T antigen genome, based on immunostaining analysis (Figure 6). The degree of protein expression is multi-factional involving transcription, translation, protein stability, and degradation. The SV40 T antigen levels in rapidly growing polyclonal cell lines appear to be regulated primarily

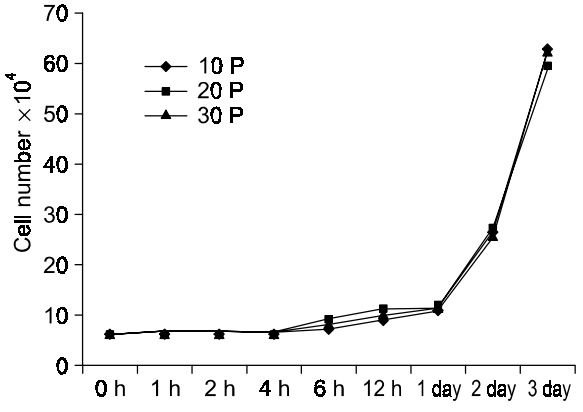

Figure 3. Growth rate assay of the midbrain tumor cell line. At 10, 20, and 30 passages, cells were recovered after 0, 1, 2, 4, 6, 12, 24, 48, and 72 h, and were stained with methyl blue for cell counting using a hemocytometer. P: passage

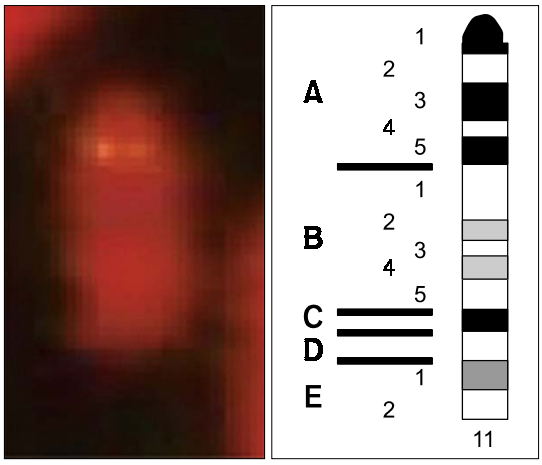

Figure 4. FISH analysis for detection of the SV40 T antigen gene in chromosomes. The yellow bar indicates the site of SV40 T antigen integration, detected on chromosome 11, A2.

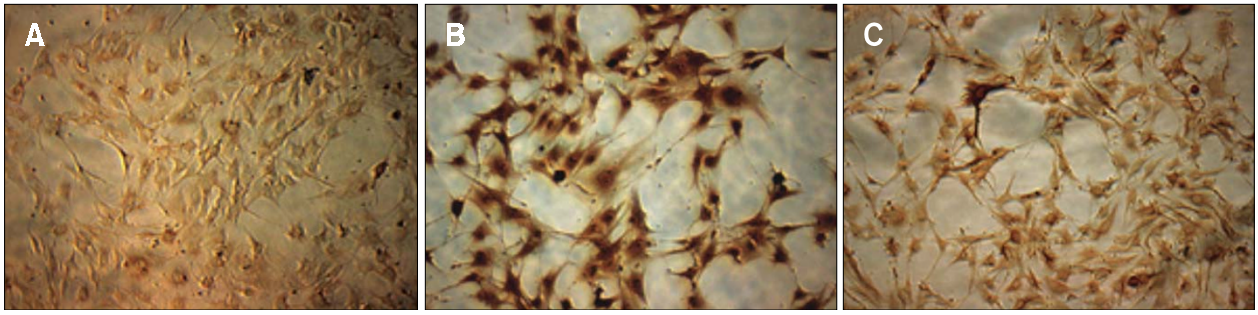

Figure 5. Characterization of cells derived from the midbrain tumor by immunostaining analysis. Cells after 30 passages were stained with GFAP (90% positive cells) and NF 150 antibody (10% positive cells). (A) control, (B) astrocyte cell - GFAP antibody, (C) neuronal cell - NF150 antibody. × 200

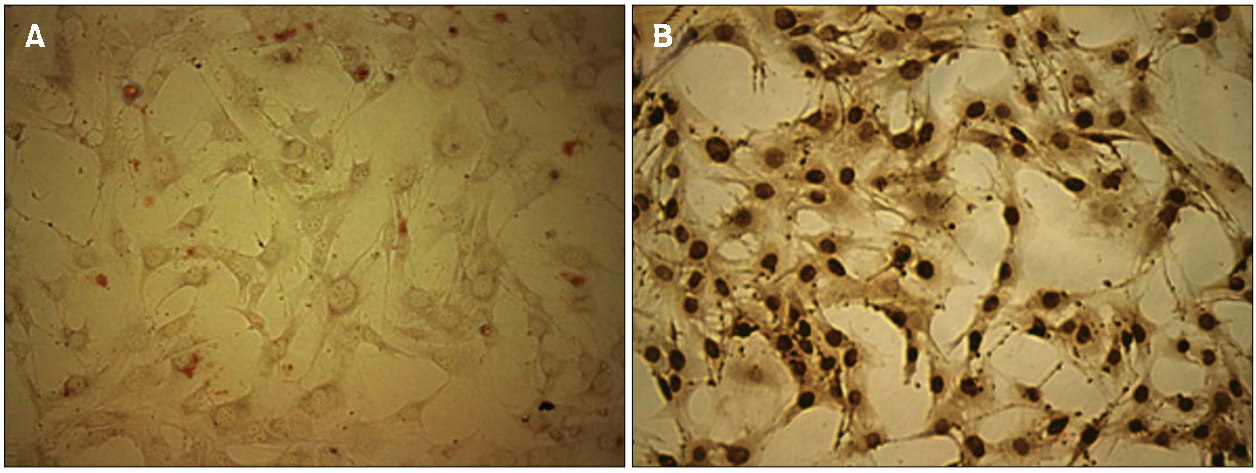

Figure 6. Culture of cells derived from the midbrain tumor. Tumor cells were reacted with the antibody to SV40 T antigen. Brown regions indicate immunoreactivity. (A) control, (B) stained with SV40 T antigen. × 200

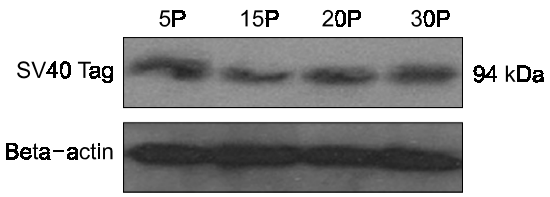

Figure 7. Identification of SV40 T antigen expression in the midbrain tumor cell line. Equally loaded protein samples from each passage of the midbrain tumor cell line were electrophoresed, blotted, and reacted with a monoclonal antibody Pab 101 against the SV40 T antigen. A major band of the expected 94 kDa protein was detected. All passages of the cell line expressed the SV40 T antigen protein. P: passage.

through transcription (Sladek *et al.*, 1992; Dolcetti *et al.*, 2003). The positive results for SV40 T antigen expression in cultured cells after 30 passages revealed that the SV40 T antigen was expressed at the same level in cells of both earlier passages (5) and later passages (10, 20, and 30), and that the SV40 T antigen gene was activated during this culture period (Figure 7).

Discussion

We generated six transgenic mice by using the vasopressin gene construct to drive the expression of SV40 T antigen (Figure 1), which manifests a transforming potential through probable inactivation of the major cellular tumor suppressor protein p53 (Symonds *et al.*, 1993; McCarthy *et al.*, 1994; Symonds *et al.*, 1994; Seo *et al.*, 2004). Five of the founder mice died between 2 and 6 weeks of age without transmitting the transgene to offspring (Lee *et al.*, 2003). These mice became lethargic and severely dehydrated before death. It is possible that specific expression of the SV40 T antigen in the hypothalamic magnocellular neurons of pVPSV.IGR3.6 transgenic mice may have a deleterious effect on hypothalamic development. VP is synthesized in the

nuclei of the hypothalamic magnocellular neurons. In this study, however, tumor production was identified in the midbrain and not in hypothalamic-derived cells. The appearance of ectopic tumors in transgenic mice may be due to lack of tissue specificity of the transgene that was used (Montag *et al.*, 1993; Chen *et al.*, 2002).

However, at least two different transcripts for the VP promoter and SV40 T antigen fragments have been expressed through alternate pVPSV.IGR3.6 gene usage and alternative splicing (Lee *et al.*, 2003). An alternate and less likely explanation would be that the VP promoter has nothing to do with tissue-specific expression of the transgene. This is unlikely because the SV40 early gene sequence itself, without an inherent or heterologous promoter element, does not induce tumors of any kind (Hanahan *et al.*, 1985, Messing *et al.*, 1985). Therefore, the coding sequences of the transgenes were examined to determine if they could be normally expressed in the transfected cell lines. Since VP derived cell lines were not available, the VP promoter was replaced with the CMV-β universal promoter, then transfected into the neuronal cell line PC-12. The SV40 T antigen was detected in the nucleus of PC-12 neuronal cells by means of immunohistochemistry using a monoclonal antibody (Pab101) against the SV40 T antigen (data not shown).

The 3.6 kb IGR fragment (designated pVPSV.IGR3.6) included a 3.6 kb section of the 3´ flanking region that was integrated in chromosome 11, A2. Histological studies of the tumors revealed PNETs in the midbrain, and a high SV40 T antigen protein expression level was also identified in the tumors by immunohistochemistry and Western blotting (Figure 6 and 7). These results suggest that SV40 T antigen expression under the control of the VP gene can affect morphological changes in the transgenic brain that, in turn, eventually cause PNETs. PNETs of the central nervous system are a family of brain tumors that account for 20 % of pediatric midbrain tumors (Park *et al.*, 1983). They are characterized by primitive histological features resembling neuroepithelial stem cells from the embryonic neural tube or immature progeny. Therefore, pVPSV.IGR3.6 mice provide a model system for investigating PNET tumorigenesis in the midbrain. This midbrain tumor cell line is composed of astrocytes and neuronal cells.

We have explored the relationship between SV40 T antigen expression as a function of cell growth, and cell differentiated morphologies of SV40 T antigen established mouse astrocytes and neuronal cells. These cells may depend upon expression of the T antigen for cell growth and cell differentiated morphologies (Figure 2 and 3). This result suggests that the inactivated pRb pathway is under selective pressure for deregulation of Akt, an event that may further facilitate astrocytoma development by a reduction in related apoptosis (Xiao *et al.*, 2002). These cells exhibited similar growth and culture morphology characteristics in both earlier and later passages. It is speculated that 30 passages are insufficient to reach crisis and immortalization. The SV40 T antigen is known to interact with anti-oncogene products, such as p53 and Rb, and may be involved in deregulation of genes for proliferation and differentiation. This may lead to a loss of systemic dependence on growth factors, indication of autocrine loops, up-regulation of growth factor receptors, and resistance to apoptose (Tognon *et al.*, 1996, Kim *et al.*, 2001). Thus, midbrain tumor cells will be useful for further study of tumorigenesis, replenishment, and differentiation of astrocytes and neuronal cells (Figure 5).

The integration sites of the transgene may have contributed to the spatial distribution of the midbrain tumor. This cell line may also be valuable for study of midbrain tumorigenesis, replenishment, and differentiation of neural cells.

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