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Similarity on neural stem cells and brain tumor stem cells in transgenic brain tumor mouse models

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Research Highlights

- (1) Previous studies have shown that tumor stem cells exist in brain tumor tissue, but their source is unclear. Some scholars presumed that these cells originated from neural stem cells.
- (2) This study established stable doxycycline-inducible transgenic mouse brain tumor models (c-myc⁺/SV40Tag⁺/Tet-on⁺) to study the characterization of brain tumor stem cells.
- (3) Results found that doxycycline-induced neural stem cells in the subventricular zone of transgenic mice have increased proliferative activity and inhibitory differentiation similar to tumor stem cells, suggesting that neural stem cells have a tendency for malignant transformation, and could be a source of tumor stem cells.

Abstract

Although it is believed that glioma is derived from brain tumor stem cells, the source and molecular signal pathways of these cells are still unclear. In this study, we used stable doxycycline-inducible transgenic mouse brain tumor models (c-myc⁺/SV40Tag⁺/Tet-on⁺) to explore the malignant transformation potential of neural stem cells by observing the differences of neural stem cells and brain tumor stem cells in the tumor models. Results showed that chromosome instability occurred in brain tumor stem cells. The numbers of cytolysosomes and autophagosomes in brain tumor stem cells and induced neural stem cells were lower and the proliferative activity was obviously stronger than that in normal neural stem cells. Normal neural stem cells could differentiate into glial fibrillary acidic protein-positive and microtubule associated protein-2-positive cells, which were also negative for nestin. However, glial fibrillary acidic protein/nestin, microtubule associated protein-2/nestin, and glial fibrillary acidic protein/microtubule associated protein-2 double-positive cells were found in induced neural stem cells and brain tumor stem cells. Results indicate that induced neural stem cells are similar to brain tumor stem cells, and are possibly the source of brain tumor stem cells.

Key Words

neural regeneration; stem cells; neural stem cells; brain tumor stem cells; subventricular zone; brain tumor; transgenic mouse model; multidirectional differentiation; doxycycline; neuroregeneration

INTRODUCTION

The tumor stem cell hypothesis that tumors arise from cells that exhibit the ability to self-renew as well as give rise to differentiated tissue cells that are responsible for

tumor progress was proposed in recent years^[1-2]. Tumor stem cells have been isolated from tumors of various systems, such as hematologic and central nervous system tumors^[1-5]. Gliomas are the leading cause of cancer mortality and remain difficult to cure despite advances in surgery and adjuvant

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therapy. The mechanism of brain tumorigenesis remains unclear. There is overwhelming evidence that brain tumors arise from cells with the ability for proliferation and differentiation^[6-12]. Although brain tumor stem cells have been identified, their source and molecular signal pathways are still not fully understood^[7]. It is believed that brain tumor stem cells are the foundation of the occurrence and maintenance of gliomas^[6-7, 9]. However, more evidence is needed to support this theory, such as the origin of brain tumor stem cells, the molecular basis of brain tumor stem cells (transformation and differentiation), and targeted treatment. The potential of neural stem cells to transform into brain tumor stem cells has long been considered^[3-5], but has not been confirmed. Tumor stem cells play an important role in resistance to radiotherapy and chemotherapy^[13-14], angiogenesis^[15] and metastasis^[16]. It is therefore important to analyze the properties of brain tumor stem cells and neural stem cells from brain tumor specimens to study the mechanism and treatment strategies.

Animal models are essential for studying the mechanism of tumor development and treatment strategies. Transgenic mouse brain tumor models are the ideal model for investigating cancerous tumors^[17] and they comply with the natural order of tumors, including the relationship between tumor and host. They are also good models for examining tumor occurrence and microenvironmental factors^[18]. The key for this model is choosing suitable genes to induce tumors. The SV40Tag transgenic animal model is widely used, but its brain tumor incidence is low^[11]. The c-myc pathway is one of the most important mechanisms of glioma^[19].

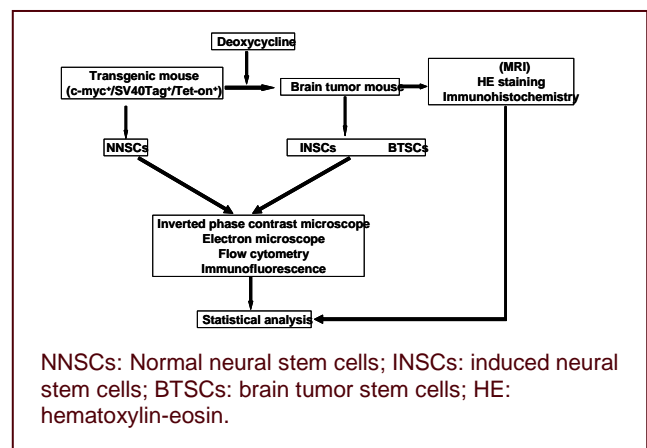
This study established a doxycycline-inducible transgenic brain tumor model (c-myc⁺/SV40Tag⁺/Tet-on⁺) in mice that was stable and controllable^[20]. The mouse brain tumor model formed was medulloblastoma, which is a form of malignant glioma that has more stem cells than other brain tumors^[21-22]. Normal neural stem cells, induced neural stem cells and brain tumor stem cells were isolated from the corresponding transgenic mouse models. The properties of the three types of cells were detected by inverted phase contrast microscopy, electron microscopy, flow cytometry, and immunofluorescence to explore the malignant transformation potential of neural stem cells.

RESULTS

Experimental procedure analysis

The Tet-on, pTRE2-SV40Tag female transgenic mice

were mated with pTRE2-c-myc transgenic male mice. Gliomas were induced in the offspring (c-myc⁺/SV40Tag⁺/Tet-on⁺) with doxycycline. Normal neural stem cells were isolated from the subventricular zone tissue of the non-induced transgenic mouse (c-myc⁺/SV40Tag⁺/Tet-on⁺), and induced neural stem cells and brain tumor stem cells were isolated from the subventricular zone tissue and tumor tissue of the induced transgenic mouse (c-myc⁺/SV40Tag⁺/Tet-on⁺). Experimental flow graph is listed as follows:



Identification of brain tumor mouse models

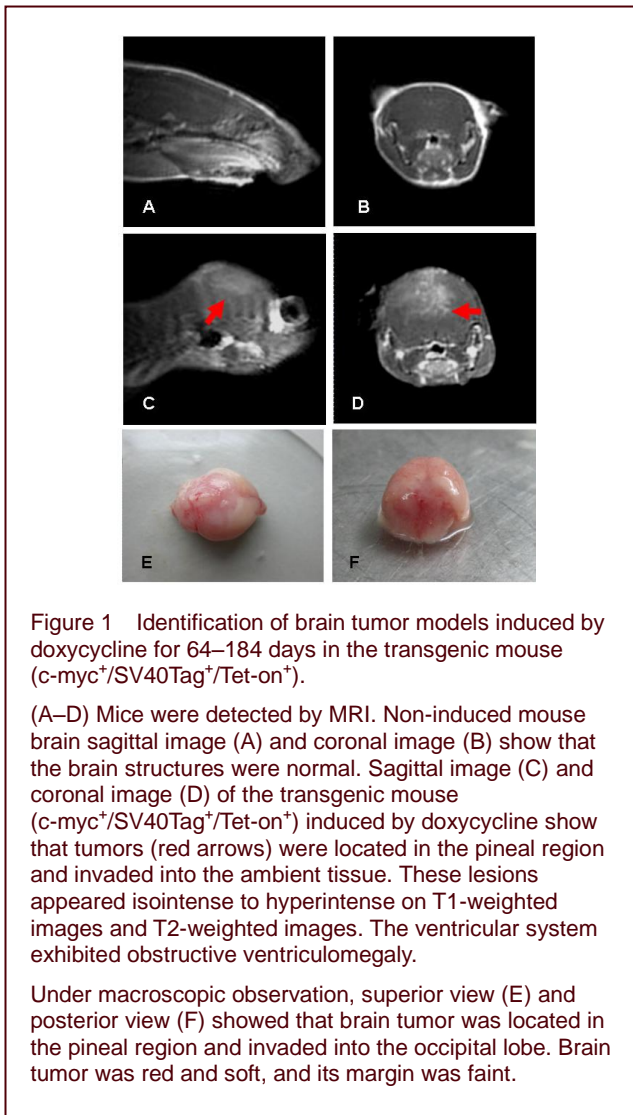
Tumors were detected in mice using MRI. Non-induced mouse brain images showed that their brain structures were normal. Images of transgenic mouse (c-myc⁺/SV40Tag⁺/Tet-on⁺) models induced by doxycycline showed that tumors were located in the pineal region and invaded the surrounding tissue. These lesions appeared isointense to hyperintense on T1-weighted images and T2-weighted images. These lesions showed homogeneous or heterogeneous enhancement in the contrast images. The ventricular system was obstructive ventriculomegaly (Figure 1A–D).

Under macroscopic observation, brain tumor was located in the pineal region that invaded the occipital lobe. Brain tumor was red and soft, and its margin was faint (Figure 1E, F).

Immunohistochemical results indicate that mice induced by doxycycline began expressing c-myc and SV40Tag (Figure 2A, B). After tumor formation, c-myc and SV40Tag were overexpressed (Figure 2C, D), and tumor cells overexpressed the stem/progenitor cell marker nestin (Figure 2E).

Hematoxylin-eosin staining showed that the brain tumor was undifferentiated medulloblastoma, which was highly invasive and exhibited low differentiation. Hemorrhage,

necrosis, heteromorphism and caryocinesia were also manifested (Figure 2F).



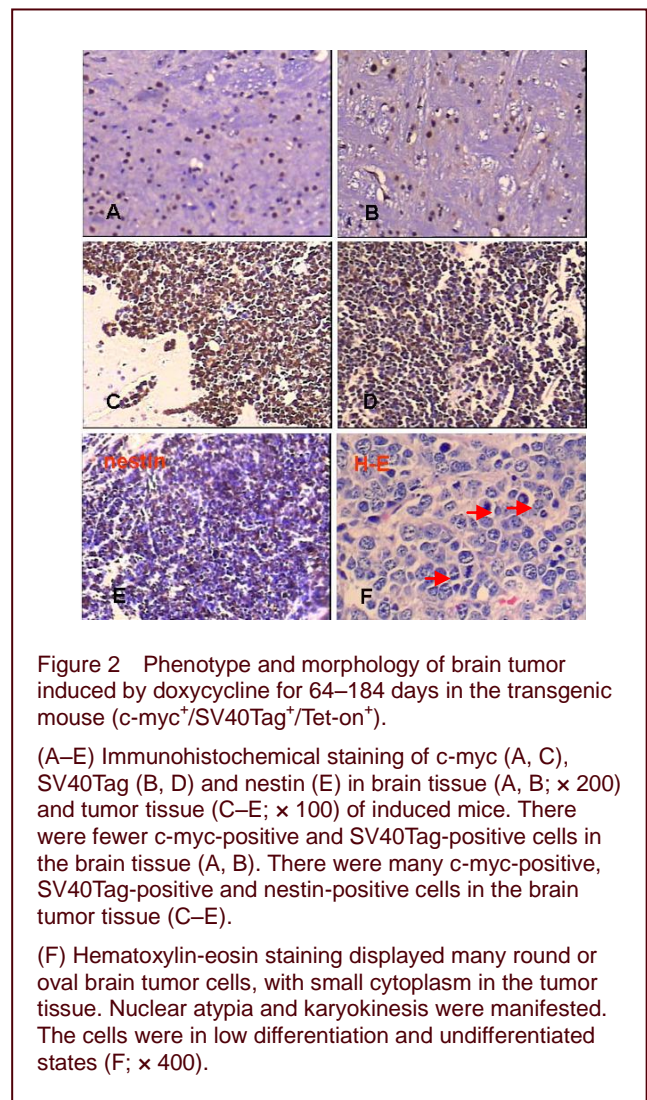
Identification of normal neural stem cells, induced neural stem cells and brain tumor stem cells

Normal neural stem cells, induced neural stem cells and brain tumor stem cells were cultured in Dulbecco's modified Eagle's medium/F-12 medium under the same conditions. 5-bromo-2'-deoxyuridine (BrdU) was added into the medium. After 3 days *in vitro*, all three cell types were positive for nestin and BrdU. Brain tumor stem cells exhibited strong positive staining for nestin (Figure 3A). The nuclear-cytoplasmic ratio of all three cell types was high, but cellular organs associated with differentiation and degradation, such as cytolysosomes, were less in brain tumor stem cells and induced neural stem cells than in normal neural stem cells (Figure 3B).

Proliferation of normal neural stem cells, induced neural stem cells and brain tumor stem cells

Under inverted phase contrast microscopy, the ap-

pearance of the three types of stem cell sphere is similar at the beginning, showing small and uniform cell spheres. During the following days in culture, induced neural stem cell and brain tumor stem cell spheres grew significantly faster and reached a bigger size than normal neural stem cell spheres (Figure 4A). At 7 days *in vitro*, the proliferation of normal neural stem cells reached a peak, with a diameter of $120 \pm 8.66 \mu\text{m}$. In contrast, induced neural stem cells and brain tumor stem cells proliferated continually and the diameter of cell spheres was about $360 \pm 13.62 \mu\text{m}$ and $380 \pm 21.46 \mu\text{m}$, respectively (Figure 4B).



At 14 days *in vitro*, flow cytometry indicated that the proliferation index was brain tumor stem cells > induced neural stem cells > normal neural stem cells (Figure 5A, B). All normal neural stem cells and induced neural stem cells were diploid, while the percentage of diploid cells in brain tumor stem cells was 73.23 ± 7.8 and other cells were aneuploid; chromosome instability appeared in brain tumor stem cells (Figure 5C).

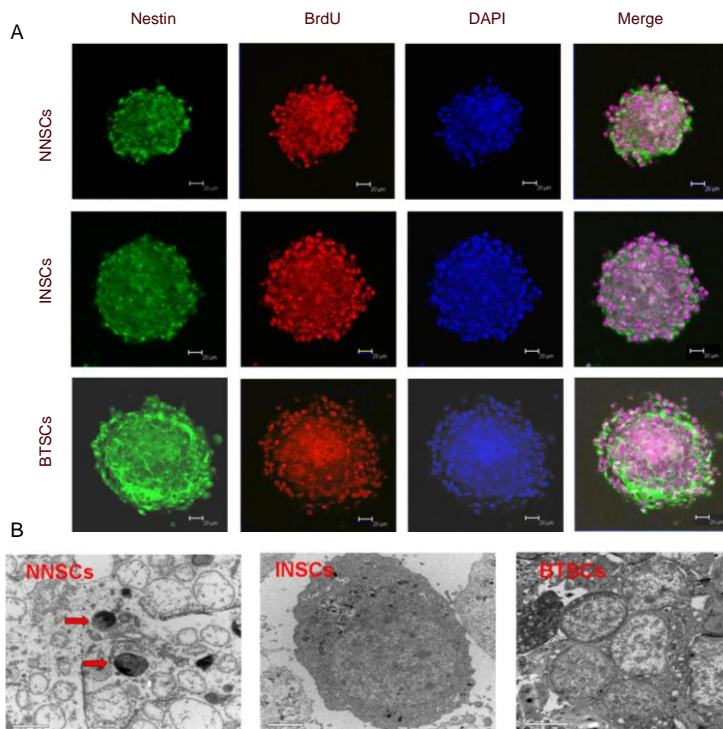


Figure 3 Identification of normal neural stem cells (NNSCs), induced neural stem cells (INSCs) and brain tumor stem cells (BTSCs).

NNSCs and INSCs were derived from the subventricular zone tissue of non-induced and induced transgenic mice. BTSCs were derived from brain tumor tissue of the induced transgenic mouse.

(A) After 3 days *in vitro*, NNSCs, INSCs and BTSCs were detected by nestin (green), 5-bromo-2'-deoxyuridine (BrdU) (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue) (immunofluorescence, inverted phase contrast microscope, bars: 20 μ m). All of them were nestin/BrdU/DAPI triple-positive. BTSCs were strongly stained for nestin.

(B) After 14 days *in vitro*, NNSCs, INSCs and BTSCs were observed by electron microscopy. The nuclear-cytoplasmic ratio of the three types of cells was high, the number of cytolysosomes (red arrows) of NNSCs ($\times 20\ 000$) was greater than that of BTSCs ($\times 5\ 000$) and INSCs ($\times 10\ 000$).

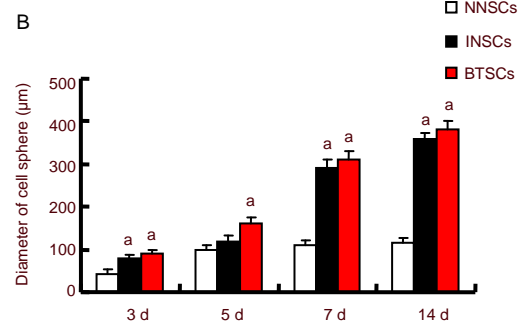
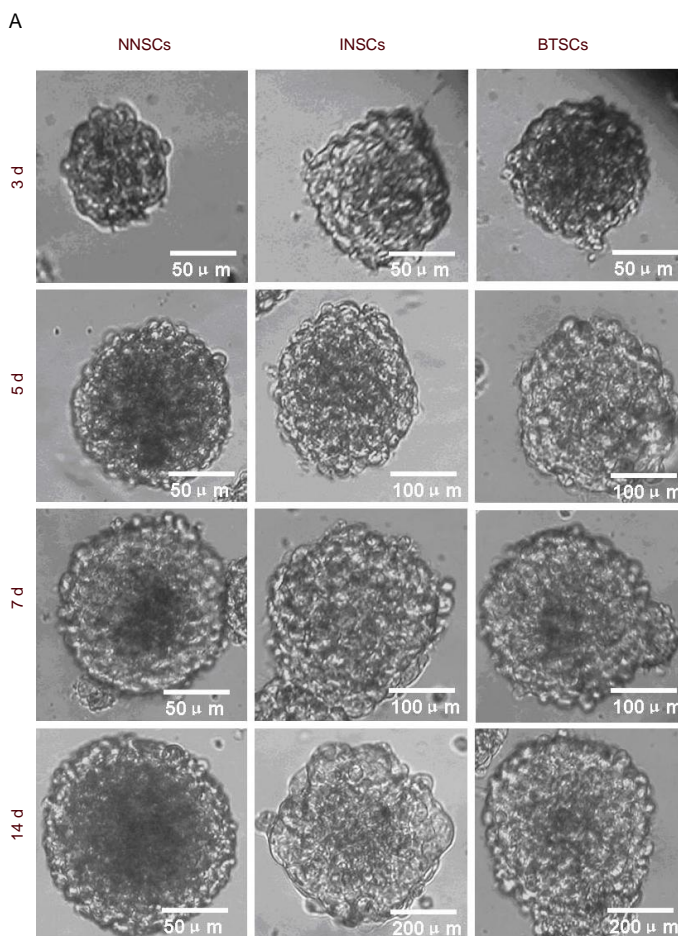


Figure 4 Proliferation of normal neural stem cells (NNSCs), induced neural stem cells (INSCs) and brain tumor stem cells (BTSCs) under inverted phase contrast microscope at 3, 5, 7, 14 days *in vitro*.

NNSCs and INSCs were derived from the subventricular zone tissue of non-induced and induced transgenic mice. BTSCs were derived from brain tumor tissue of the induced transgenic mouse.

(A) The appearance of spheres of the three types of stem cells was similar at 3 days *in vitro*, showing small and uniform cell spheres. In the following days in culture, INSC and BTSC spheres grew significantly faster and reached bigger sizes than NNSC spheres.

(B) The diameter of NNSC, INSC and BTSC spheres. The data are expressed as mean \pm SD, $n = 6$. ^a $P < 0.05$, vs. NNSCs. One-way analysis of variance followed by Dunnett's test was performed.

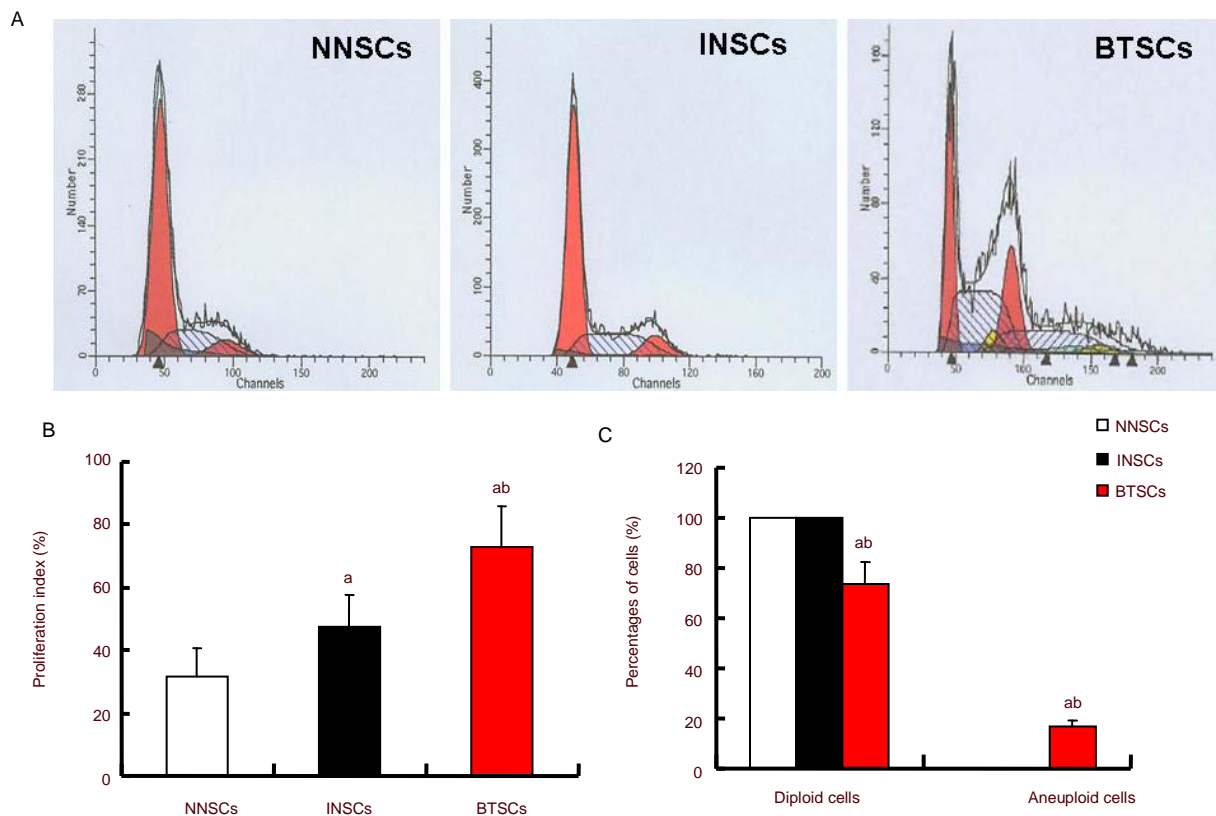


Figure 5 Normal neural stem cell (NNSC), induced neural stem cell (INSC) and brain tumor stem cell (BTSC) proliferation detected by flow cytometry at 14 days *in vitro*.

NNSCs and INSCs were derived from subventricular zone tissue of non-induced and induced transgenic mice. BTSCs were derived from brain tumor tissue of the induced transgenic mouse.

(A) Images of flow cytometry. The first peak (red) represents the number of G₀ (G₁) cells. The second peak (red) represents the number of G₂ (M) cells. The blue grid represents the number of S cells. The yellow grid represents the number of aneuploid cells.

(B) Proliferation index was calculated by $G_2(M) + S / G_0(G_1) + G_2(M) + S$.

(C) Percentage diploidy in NNSCs, INSCs and BTSCs.

The data are expressed as mean \pm SD, $n = 6$; ^a $P < 0.05$, vs. NNSCs; ^b $P < 0.05$, vs. INSCs. One-way analysis of variance followed by Dunnett's test was performed.

Differentiation of normal neural stem cells, induced neural stem cells and brain tumor stem cells

Fetal bovine serum was used for differentiation culture of normal neural stem cells, induced neural stem cells and brain tumor stem cells. After 7 days *in vitro*, three types of stem cells exhibited multidirectional differentiation and all of them could differentiate into microtubule associated protein-2-positive neurons and glial fibrillary acidic protein-positive astrocytes. Microtubule associated protein-2-positive neurons and glial fibrillary acidic protein-positive astrocytes derived from normal neural stem cells were negative for nestin, with good cell morphology, showing extensive processes and rounded bodies. Microtubule associated protein-2-positive neurons and glial fibrillary acidic protein-positive astrocytes derived from

induced neural stem cells and brain tumor stem cells were positive for nestin with unclear cell morphology (Figure 6A). Abundant microtubule associated protein-2/glial fibrillary acidic protein double-positive cells were originated in the induced neural stem cells and brain tumor stem cells; the differentiated cells were poorly differentiated (Figure 6B).

DISCUSSION

Currently, most basic science research of brain tumors focuses on the cellular level. Animal models are essential for studying tumor mechanism and treatment strategies.

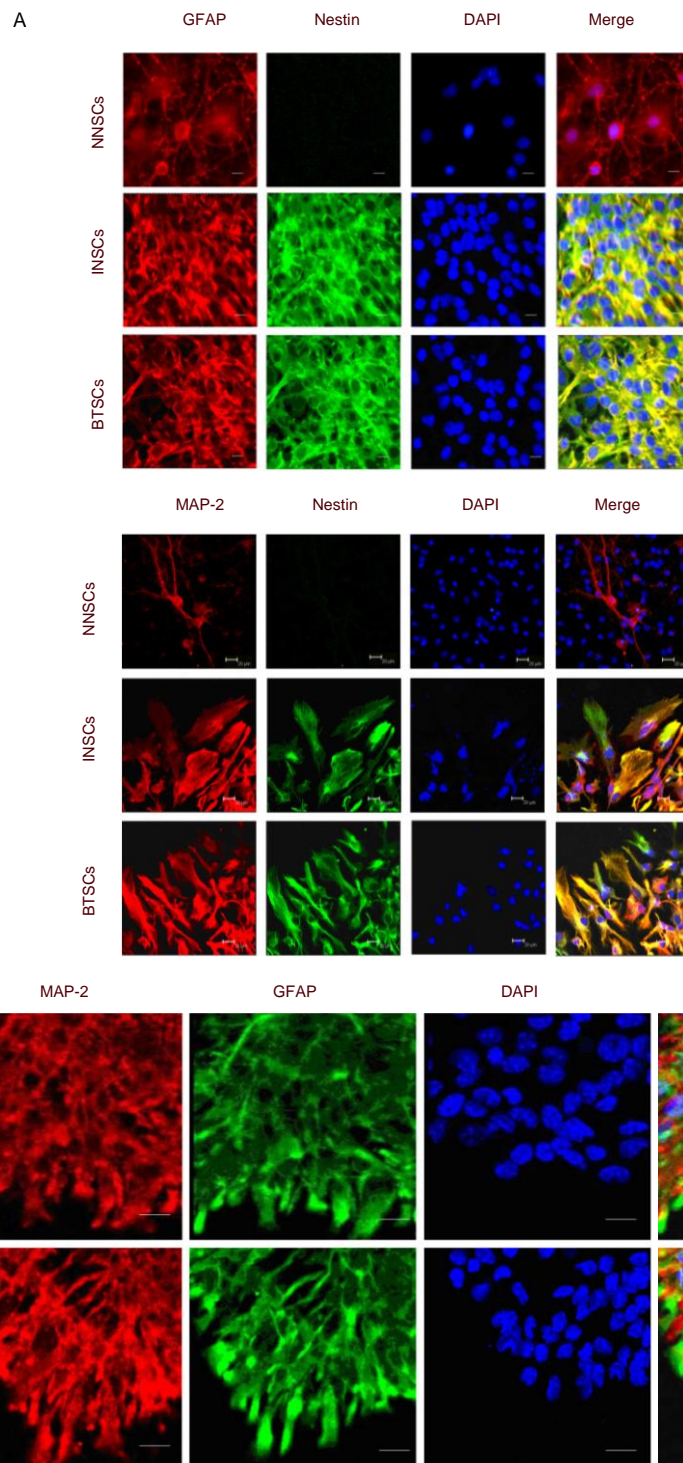


Figure 6 Differentiation of normal neural stem cells (NNSCs), induced neural stem cells (INSCs) and brain tumor stem cells (BTSCs) detected by immunofluorescence after 7 days of differentiation culture with fetal bovine serum (scale bars: 20 μ m).

NNSCs and INSCs were derived from subventricular zone tissue of non-induced and induced transgenic mice. BTSCs were derived from brain tumor tissue of the induced transgenic mouse.

(A) Microtubule associated protein-2 (MAP-2) (red) positive neurons and glial fibrillary acidic protein (GFAP) (red) positive astrocytes derived from NNSCs were negative for nestin (green). MAP-2-positive neurons and GFAP-positive astrocytes derived from INSCs and BTSCs were positive for nestin.

(B) MAP-2-positive cells derived from INSCs and BTSCs were positive for GFAP; the differentiated cells were poorly differentiated.

Xenogenic or autogeneic transplantation tumor models are widely investigated^[22-24]. These models are reproduced easily, but there are obvious disadvantages, as they do not consider the onset of cancer formation, the tumor microenvironment or the relationship between host and tumor^[17]. The regulated transgenic animal tumor model is an ideal model for basic science research^[18]. The key for this model is to choose suitable genes.

The molecular events occurring in glioma stem cells are mainly focused on the c-myc pathway. SV40Tag transgenic animal models with highly malignant tumor cells develop into medulloblastoma, the mechanism of which is inhibition of p53 and Rb by the SV40Tag, but the tumor incidence is low and unstable^[11, 25-29].

In this study, we used a doxycycline-inducible transgenic mouse brain tumor model (c-myc⁺/SV40Tag⁺/Tet-on⁺) that was stable and controllable. The mouse brain tumor was a medulloblastoma that was a malignant glioma. Medulloblastoma is the most common malignant brain tumor of children. Recent experiments have confirmed that some of the subtypes of medulloblastoma may originate from stem cells, while others result from granule neuron precursor cells, but the definition of these subtypes is still unclear^[30-32].

Although the treatment of medulloblastoma has improved, the prognosis remains poor. Almost half of all patients die, and those who survive often suffer from severe adverse effects, including cognitive impairment, endocrine abnormalities and susceptibility to secondary tumors^[33-34]. Effective treatment will most likely come from understanding the molecular and cellular origin of the tumor. Nevertheless, the origin has been debated for many years. Currently, medulloblastoma is classified as an embryonal tumor with primitive neuroectodermal tumor^[35]. Various studies showed that medulloblastoma cells express stem cell markers, such as p75^{NTR}, TrkC, Zic1 and nestin, and can differentiate into neurons and glial cells^[36-39].

Recently, tumor stem cells have been isolated from the majority of brain tumors and the higher the degree of malignancy, the greater possibility and ease of separation of tumor stem cells^[40]. Some researchers hypothesize that the tumor originates from multi-potential neural stem cells. In the present study, brain tumor stem cells with the capacity of self-renewal and multidirectional differentiation potential were isolated from brain tumor tissue. However, their differentiating activity was poor,

while their proliferating capacity was good. Aneuploidy and chromosome instability were found in brain tumor stem cells.

To gain a better understanding of the behavior of the tumor, it is important to determine the exact origin of the tumor cells. Neural stem cells generally have similar characteristics as tumor stem cells in the central nervous system, including strong proliferative potential and differentiation capacity. They also respond to the regulation of cellular pathways in many brain tumors, such as Sonic hedgehog, Notch, Wnt, B-lymphoma mouse Moloney leukemia virus insertion region 1, phosphatase and tensin homologue deleted on chromosome ten, c-myc, and p53^[36]. Evidence shows that the changes of regulation of these pathways could cause tumors^[41-42].

In this study, brain tumor stem cells and neural stem cells were isolated and identified successfully from induced and non-induced mouse tumor models. The reproductive activity of induced neural stem cells was better than normal neural stem cells and cellular organs associated with differentiation and degradation, such as the cytolysosome and autophagosomes, were reduced in induced neural stem cells compared with normal neural stem cells. Glial fibrillary acidic protein-positive and microtubule associated protein-2-positive cells from induced neural stem cells were positive for nestin. These results indicate that the reproductive activity of induced neural stem cells was enhanced and differentiation ability was impaired; the differentiated cells were poorly differentiated, which were similar to brain tumor stem cells.

The results of the present study indicate that neural stem cells of the subventricular zone in the induced mouse tumor model acquired early malignant transformation *in vitro*. However, the changes that occur in them *in vivo* are still unclear. For example, whether neural stem cells of the subventricular zone in the induced mouse tumor model could contribute to glioma formation or migrate into the diseased region and what factor is associated with the progress remain to be understood. Taken together, this study investigated the biological properties of induced neural stem cells *in vivo*.

In conclusion, our preliminary findings show homology between brain tumor stem cells and induced neural stem cells, which indicate that neural stem cells of the subventricular zone acquired early malignant transformation in the induced mouse tumor model.

MATERIALS AND METHODS

Design

Animal experiment *in vivo* and cytological experiment *in vitro*.

Time and setting

The experiment was performed at the Department of Neurosurgery, the Second Affiliated Hospital of Nanjing Medical University, China, from October 2010 to October 2012.

Materials

Forty Tet-on, pTRE2-SV40Tag transgenic female mice and 20 pTRE2-c-myc transgenic male FVB mice, weighing 180–200 g, were provided by Suzhou Beldar Biological Co., Ltd. (license No. SYXK2005-0001). All animal tests were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

Methods

Establishment and identification of mouse tumor models

Tet-on, pTRE2-SV40Tag transgenic mouse and pTRE2-c-myc transgenic mouse were fed in micro ventilation cages in a specific-pathogen free animal room with no more than five mice/cage, temperature $24 \pm 1^\circ\text{C}$, relative humidity of $70 \pm 4\%$, with free access to water and a 12-hour light/dark cycle. The Tet-on, pTRE2-SV40Tag female transgenic mice and pTRE2-c-myc transgenic male mice were mated in a 2:1 ratio. Pregnant female mice were individually subcaged until weaning. Doxycycline (0.5 mg/mL) (Sigma, St. Louis, MO, USA) was added to the drinking water of the offspring. The water was replaced every other day. Mice with tumor symptoms were identified by MRI (Achieva 1.5 T; Philips, Amsterdam, the Netherlands), hematoxylin-eosin staining, and c-myc/SV40Tag/nestin immunohistochemistry as described previously^[18].

Isolation and culture of normal neural stem cells, induced neural stem cells and brain tumor stem cells

Under anesthesia with chloral hydrate (2 mL/kg, intraperitoneally), normal neural stem cells and induced neural stem cells were derived from the subventricular zone tissue of non-induced and induced transgenic mice. Brain tumor stem cells were derived from brain tumor tissue of induced transgenic mice. Three types of tissue were mechanically dissociated into a single-cell suspension by 0.25% trypsin digestion. Cell number and viability were determined by staining a small volume of cell sus-

pension with 0.4% trypan blue. Cell suspension was then plated onto culture flasks at a density of 4×10^4 cells/mL and maintained in Dulbecco's modified Eagle's/F-12 medium (1:1; Gibco, Carlsbad, CA, USA) containing 2% B27 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 ng/mL epidermal growth factor (Invitrogen) and 10 ng/mL basic fibroblast growth factor (Gibco). To label the dividing cells, BrdU (Boster, Wuhan, China) was added into the medium at a concentration of 5 nmol/L for 24 hours. Cells were maintained at 37°C in a 95% air/5% CO_2 humidified atmosphere incubator. Stem cell spheres were characterized by immunocytochemistry against anti-BrdU antibody (1:400; Diagnostics, Penzberg, Germany) and anti-nestin antibody (1:100; Chemicon, Santa Cruz, CA, USA) as described previously^[43] at 3, 5, 7, 14 days *in vitro*. Cell nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) for 30 minutes at room temperature. Photomicrographs were taken by inverted phase contrast microscopy (Olympus, Tokyo, Japan) and analyzed for size of cell spheres. At 14 days *in vitro*, some cell spheres were fixed in ice-cold 2.5% glutaraldehyde and preserved at 4°C for further processing. When processing resumed, cells were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded alcohol series, embedded in Epon 812, sectioned with an ultramicrotome, and then stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (Hitachi H-600, Tokyo, Japan).

Flow cytometry analysis

At 14 days *in vitro*, some cell spheres were mechanically dissociated into a single-cell suspension by 0.25% trypsin digestion. Collected cells were washed once in cold PBS. The cells were fixed in 70% ethanol, treated with 100 mg/L RNase (Takara, Dalian, China) at 37°C for 30 minutes and stained with 50 mg/L propidium iodide (Sigma) for 30 minutes. The cells were analyzed using flow cytometry (Epics XL; Beckman Coulter, Fullerton, CA, USA).

Induction of normal neural stem cell, induced neural stem cell and brain tumor stem cell differentiation

All three types of cell spheres were passaged every week by dissociation of cell spheres with 0.25% trypsin digestion. After four passages, single cells (1×10^5 cells/mL) from stem cell spheres were cultured on poly-D-lysine-coated coverslips in 24-well plates in Dulbecco's-modified Eagle's/F12 medium containing 10% fetal bovine serum (Sigma) for 7 days.

Immunocytochemistry

After 7 days in culture, cells were fixed with 4% para-

formaldehyde in 0.1 mol/L phosphate buffer for 1 hour. Cells were blocked in 5% goat serum for 30 minutes at room temperature and then incubated for 12 hours at room temperature with primary antibodies. After washing three times in PBS, the cells were incubated in secondary antibodies for 6 hours at room temperature. Primary antibodies were as follows: rat anti-mouse monoclonal microtubule associated protein-2 (1:200; Chemicon), rat anti-mouse monoclonal glial fibrillary acidic protein (1:200; Sigma) or rabbit polyclonal anti-glial fibrillary acidic protein (1:800; Millipore, Billerica, MA, USA), and rabbit polyclonal anti-*nestin* (1:200; Millipore). Secondary antibodies were Alexa Fluor 568-conjugated (red) goat anti-rat IgG (1:500; Invitrogen, Carlsbad, CA, USA) or FITC-conjugated (green) goat anti-rabbit IgG (1:200; Millipore). Cell nuclei were counterstained with DAPI for 5 minutes at room temperature. Immunopositive cells were observed using an inverted phase contrast microscope (Olympus). Photomicrographs were taken and analyzed for cell phenotype.

Statistical analysis

Images were analyzed by Leica Qwin image processing and analysis software (Leica Imaging System, Cambridge, UK). Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Data were presented as mean \pm SD. Statistical comparisons were performed using one-way analysis of variance followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

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