

# Xenograft Model of Human Brain Tumor

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## 1. Introduction

In the life sciences, scientists are paying more and more attention to the human brain, the essential center of the body. The gliomas in human brains are especially unique challenges for doctors due to the difficulties of identifying early cancer lesions and little effective treatments. Gliomas account for about half of the central nervous system tumors. Due to its invasive growth and other malignant behaviors, gliomas are difficult to be radically cured; moreover, most of gliomas are difficult to be early detected, even they are discovered, it is difficult to cure them because of their resistance to radiation or chemotherapy. Therefore, developing animal models of human gliomas is essential for us to explore the mechanisms of occurrence and development of brain tumor and promote clinical research. From past to future, the far-sighted men attached and will continue to attach importance to the development of animal models of human gliomas. Judging from the research and development process, human beings have, after a hundred years' efforts, moved from the development and application of animal models of spontaneous and induced brain tumor to the generation of the experimental platform of various animal models of human brain tumors. Now we are trying to improve the simulation of the animal models to the human diseases. For the human glioma, the early model was the solid tumors formed by directly inoculating in vitro passaged cell lines to animals; then, human glioma tissue were successfully inoculated into animals. In recent years, with the progress of tumor molecular biology, transgenic or gene knockout procedures are used to generate genetic engineering mouse brain tumor model, which meet the requirement of finding the molecular etiology of human brain tumors through specific molecular genetics. After the successful cloning of glioma stem cells, the establishment of animal models retaining the characteristics of glioma stem cells is on the agenda. In short, although we face difficulty building up animal models of brain tumor, we have tried to imitate the models to diseases from the system-cell level to the system-cells -molecular level.

## 2. The types of tumor model

### 2.1 Allogeneic graft model of mouse brain tumor

Although allogeneic grafting of animals' spontaneous tumors succeeded before, this method had been abandoned because of its poor simulation such as the low incidence, the early stage occult and short survival period of tumor-bearing animals. It has been replaced by the development of induced animal brain tumor model. The most commonly used cancer-

inducing methods are by chemical carcinogen or by viruses. Back to 1939, Seligman et al reported that implantation the pill made of the polycyclic aromatic hydrocarbons methylcholanthrene in mice brain induced glioma and sarcoma. From the middle of last century, the systemic administration of pro-nerve alkylating agent was proved to induce nervous system tumors. Since then, the polycyclic aromatic hydrocarbons-induced method has been gradually replaced by nitrosourea derivatives-induced methods, especially by the methods using nitrosourea and ethyl nitrosourea which have higher rates of inducing central nervous system tumors. Nitrosourea can induce tumor effectively in adult mice. It can induce astrocytoma, oligodendrocytes, ependymal tumor or the most commonly glioma, which is the mixture of all the previous tumor cell types. The intravenous injection of ethyl nitrosourea to the 20 days pregnant rats induced the central nervous system tumors in all the offsprings. The widely used P494, C6, 9L and G422 animal glioma models are all produced by the similar methods. The sensitivity to carcinogen of rat central nervous system is formed 10 days before birth, and reaches the peak at birth (50 times sensitized than the adult). One month after birth, the sensitivity drops to the adult level.

In addition to the chemical carcinogen, oncogenic virus is also used to induce gliomas. The virus can induce two types of brain tumors: RNA viruses, such as Rous sarcoma virus or DNA viruses, such as adenovirus. Concentrated Rous sarcoma virus (0.01ml) have been injected into the brains of newborn dogs, which all had gliomas after a period of latency. (Bigner et al, 1970) It was also reported that direct injection of AD12 virus into the brain of mouse that had been born for 24 hours induced brain tumors after an incubation period of several months, most of which are medulloblastoma. Usually for the newborn rats, tumor is formed 9-100 days after birth, the longest one takes about a year to mature after viral transfection. Tumor cells were implanted by intracerebral inoculation of  $4 \times 10^7$  chick embryo fibroblasts infected with the Schmidt-Ruppin strain of Rous sarcoma virus (RSV). With a 15 to 67 day latency, brain tumors were induced in 11 (73.3%) of 15 RSV-inoculated monkeys. (Tabuchi et al, 1985) Scientist also found that inoculating the viruses isolated from brain tissue of progressive multifocal leukoencephalopathy (PML) patients into hamsters' or monkeys' brains could induce cerebellum medulloblastoma, hypothalamic gliomas, pinealomas, intraventricular ependymoma and many other types of brain tumors in various locations. Brain tumors caused by viruses can only be produced into models with stable biological characteristics by cloning, such as the RT2 glioma model induced by the chicken tumor virus.

These chemical carcinogens and oncogenic viruses are prevalent in the human environment, which imitates the natural occurrence of spontaneous human gliomas. The induced tumors in animals will be continuously passed through generations and the biological characteristics of tumors are relatively stable, which play active roles in understanding the tumor development and in preventing tumors. However, animal models of brain tumors induced by oncogenic have different cycles and different pathological types. Compared to human brain tumors, the induced brain tumors in animals were different in genetics, cell biology and histology. Researchers hope to develop the xenograft model of human brain tumor to improve the simulation.

## **2.2 Xenograft model of human brain tumor**

### **2.2.1 Animal strains**

Typically, it is impossible for the human tumors to grow in the animal due to strong immune rejection. So the tumor is usually inoculated in the anterior chamber or parts of the hamster

cheek pouch where immune cells can not reach. But these tumors have very unstable biological characteristics such as spontaneous regression, so the above method is only used for tumorigenicity testing. We have injected immunosuppressive agents dexamethasone in newborn rats to successfully inoculate the human glioma cell line SHG44 into the brain of Westar rats, which is the first experiment to use human glioma cells for in situ animal tumorigenicity experiments. Later, due to the appearance and wide usage of immune-deficient animals, the injection methods of immunosuppressive agents have been abandoned.

Internationally, there are more than 30 kinds of pure T cell deficient nude mice with clear genetic background available, as well as T cells and B cells combined deficient mice -- (Lasat), SCID (severe combined immunodeficiency), NOD-SCID, CBA / I mice, and Beige mice with T cells and NK cells double deficiencies. The nude mice currently used in China - Balb / C, Swiss, and NC strains -- were imported from abroad in the early 80s of last century. The NC strain mice introduced from Japan in 1981 are non-inbred nude mice with high reproductive rate; they are resistant to pathogens and easy to manage. They are still used for establishing human glioma models NHG-1.

The recent established green fluorescent protein (GFP) transgenic mice C57BL/6J-GFP are very popular because it is easy to trace the green fluorescence in the host tissue or cells of tumor xenograft models. However, tumor xenografts could not be established because of their normal immune function. To apply them in human cancer model transplantation, Yang and colleagues (Yang et al, 2004) successfully hybridized them with nude mice to produce immunocompromised nude mice expressing GFP which are suitable for human cancer transplantation. We have also successfully cultured the NC nude mice expressing GFP (Figure 1), and these mice have been used in human glioma xenograft experiment.

### 2.2.2 Method of transplantation

Commonly, tumor cell lines cultured in vitro, tumor tissue or the cell suspension digested from tumor tissue are used for establish xenograft model. Usually, the implantation sites can be subcutaneous space, foot, abdomen, renal capsule, intracranial brain parenchyma, ventricles or spinal subarachnoid space, depending on the experimental need. In the early stage, we established the NHG-1 solid tumor subcutaneously in nude mice model using the human brain malignant astrocytic tumor cell lines implantation, the NHE-2 nude mice xenograft model using human ependymoblastoma tissue implantation and the mouse - human chimeric immune mice model of human glioma using human peripheral blood mononuclear cells SCID transfusion. By subcutaneous xenograft, it is not only convenient to observe the tumor volume by visual or dynamic measurement, but also easy to evaluate the effects of anticancer drugs. However, the tumor formed in this way is not in the brain and the blood-brain barrier, the macro- and micro- environment of tumor cells are quite different from those in clinical diseases. Therefore, the orthotopic transplantation model of human brain glioma in nude mice is a better model for imitating the clinical diseases.

The animal model of glioma orthotopic transplantation used in previous researches usually applied cell suspension cranial injection or tissue inoculation with craniotomy (Antunes L et al, 2000, Bradley NJ et al, 1978, DeArmond SJ et al, 1994, Horten BC et al, 1981, Rana MW et al, 1977, Taillandier L et al, 2003). The former method can be used to generate the tumor model, but the procedure is too complicated. There are lots of issues, for example: (1) the tumor cells for inoculation are usually cultured in vitro for several generations, which are damaged during the trypsin digestion. It is difficult to get enough living cells; (2) the injection volume and speed are restricted by automatic pump because of the small

compensatory volume. It will take a long time to make the model; (3) the inoculation cells are out of the incubator for too long to keep all alive because the operation takes too much time. Although the same amount of cells was used in different batches of xenograft, it is hard to get about the same number of alive cells in every experiment, which have impact on the tumor-inducing rate and latency. Although the later method can avoid the above problems, there are still lots of concerns such as large craniotomy injury to mice, complicated operation and other issues. We used needle for transplanting tumor tissue in either subcutaneous or intracerebral space, as shown in Figure 2. In such way, the trauma was relatively small. Compared with the cell suspension, tissue transplantation inoculated suitable environment (stroma) at the same time. It is better in maintaining the original parental tumor structure, tumor biology or molecular phenotype.

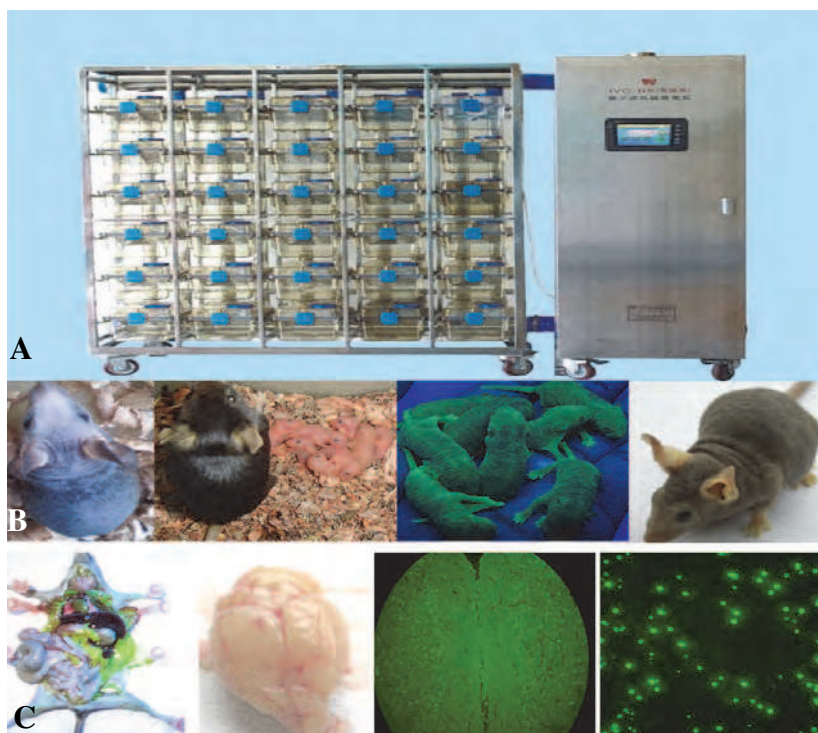


Fig. 1. The proliferation and fluorescent protein expression in nude mice transfected GFP: A row, IVC system, an independent air supply cage for mice, produced in Suzhou, consists of 4 parts: the air supply system, exhaust system, cage, mouse box. Fan is imported from German; high efficiency filter is produced by Aetna, a chinese-Japanese jointed venture; differential pressure gauge is imported from the United States. The cage is made of imported stainless steel tubes 304. The rat box is made of polysulfone transparent material. B row, from left to right are NC male mice used for breeding, neonatal of GFP/C57 female mice, bred GFP/C57/NC nude mice and adult mice. C row, from left to right are GFP/C57/NC nude mice under anatomy, eye view of mice brains, the cerebral hemispheres and bone marrow biopsy under fluorescence microscopy.

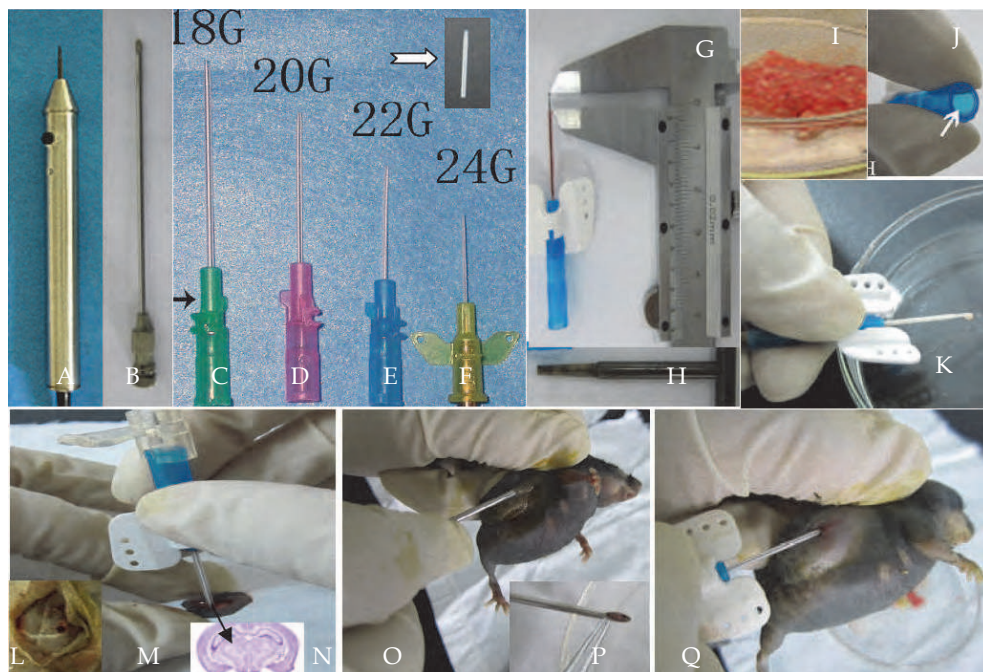


Fig. 2. The diagram of intracerebral and subcutaneous tumor xenograft transplantation: A-K: the surgical instruments, including micro-cranial drill (A), a metal trocar for subcutaneous inoculation (B), plastic trocars (C, D) and plastic trocars for intracerebral inoculation (E, F). For intracerebral inoculation, an external pin 2mm or 3mm shorter than the inner sleeve of the jacket tube (F, white arrow) must be used to control the depth of the puncture (if necessary, use the caliper G for measurement). Use the forceps to move the tumor tissue (I) on the top of the trocar (J, arrow), then use propeller (H) to push the tumor tissue into the casing, any extra tissue will overflow automatically from the needle end, and then place the core needle, push ahead to the second stent level (2-3mm length of tumor tissue is still left in the casing, at this point, K). In intracranial inoculation, the anesthetized animals should have scalp incision, and drilled at 2.5mm right to the sagittal suture, 1.0mm to the cranial coronal suture (L), the needle to be vertically inserted into the brain (2mm or 3mm) and the inner core is pushed slowly until the tumor tissue be removed from the casing into the caudate nucleus (M, N arrow). If the tissue need to be inoculated subcutaneously in the puncture site (usually in the right armpit), use ordinary needle to puncture a hole, and then put the metal casing filled with tumor tissue (P) in the puncture holes, push the core of the casing to move the tumor tissue into the subcutaneous (O), slowly remove the needle. Plastic trocar also can be used for inoculation (Q).

There are essential indicators for evaluating the inoculation quality: transplantation success rate and the stability of parent tumor characteristics. According to the author's experience, the success rate of brain tumor xenografts is determined by the following factors. (1) For the subcutaneous inoculation, the nearer the location is to the head, the easier it is to generate the model. (2) Compared with using human tumor tissue, it is easy to generate the model by using the established tumor cell lines. The *in vitro* cultured tumor cell lines have stable

biological characteristics and proliferate very fast, which make it ideal for establishing xenograft model. The tumor tissue inoculation keeps the original tumor characteristics in simulating the blood supply, interstitial structure and growth characteristics. (3) It is more successful to use tumor tissue inoculation than using trypsin digested tumor cells inoculation. The latter method can provide the exact amount of inoculation cells. (4) The more cells inoculated, the shorter the incubation period would be (no less than  $1 \times 10^7$  cells in the initial inoculation). But Singh's group (Singh et al, 2003, 2004) has reported that it was the number of tumorigenic stem cells, rather than the number of total cells, that has decisive effect. The study also showed that  $10^2$  tumorigenic stem cells are sufficient for tumor generation, while  $10^5$  non-tumorigenic stem cells would not. (5) Compared with inoculating in subcutaneous space and in the abdominal cavity, it is easier to generate the model by inoculating in the peri-renal adipose capsule with relatively low immunity or in intracerebral space.

To determine the stability of transplantation tumor biologic characteristics, the models must be evaluated by the following indicators: (1) maintain the genetic characteristics: the chromosome of transplanted tissue is same as the primary inoculated human tumor tissues; (2) maintain the morphological features: the morphology, mitotic status, tumor stroma and vascular structures of transplanted tissue are identical to the primary inoculated human tumor tissues under the light and electron microscopy; (3) maintain the tumor markers: the specific qualitative biochemical indicators or quantitative biochemical indicators of transplanted tumor tissues are consistent with those of the primary inoculated human tumor tissues; (4) maintain the stability of the biological characteristics of tumor-bearing animals: after several generations, when the transplant success rate reaches 100%, there are less difference in spontaneous regression, tumor size and the survival rate between tumor-bearing animals; (5) maintain the proliferation kinetics of tumor cells: the mitotic index, phase, cell cycle and doubling time are almost the same. According to the above criteria, we have successfully established a primary malignant glioma and lung cancer brain metastases orthotopic transplantation nude mice models.

With the progress in the cancer stem cell research, it seems the quality control of animal models should be focused on the probability of replicating the parent tumor stemness characteristics. As is mentioned above (Singh et al, 2003, 2004), Singh's group found that there are inconsistent cellular renewal and proliferation rate in human brain tumors. They have isolated CD133+ tumor stem cell, the only subtype that can cause intracranial tumors, and proved that CD133+ cells are the tumorigenic cells in human brain. These findings make the generation of stem cell-induced brain tumor animal model possible. XioNan Li's group had generated the real animal models using human brain stem cells (Shu et al, 2008). 17 cases of pediatric glioma specimens were grounded into single cells or 3-5 cells suspension, and were injected into the NOD-SCID mice brain or cerebellum using the needle. He built 10 animal models for human glioma xenograft. Evaluated by HE staining and the critical markers, especially CD133 immunohistochemistry, the transplanted tumor retained the parental tumor characteristics, like invasion, histological type and BTSCs and stem cell pool expressing CD133+. In the tumor neovascular, the human specific CD31 and CD34 were negative, while the human /mouse shared vWF was positive. The results indicated that the tumor neovascular was provided by the host. It is controversial, because previous report indicated that BTSCs provide the neovascular. BTSCs originated angiogenesis have been found *in vitro* and *in vivo*. Our results were different from those of Li' team, because we used

tissue inoculation while Li et al. used single-cell or 3-5 cells cluster inoculation; these two methods may provide different micro-environment for BTSCs. We think that tissue transplantation will better maintain the characteristics of origin tumor.

### **2.3 Genetic engineering model of mouse brain tumor**

In 1984, Brinster et al fused the promoter (MK) with polyomavirus SV40 gene. After enzyme digestion, he microinjected the DNA fragments into male pronucleus of fertilized eggs to built transgenic mice. The transgenic mice developed a variety of tumors, such as choroid plexus papilloma, thymoma and tumors in endocrine system. In 1988, Reynold et al. used H-2kb/ SV40 infusion to generate the transgenic mice, which provided results similar to Bringster's. Later, Vogel et al fused HTIV-LTR with tat gene, the injected transgenic mice can spontaneously develop neurofibroma. In 1990s, the transgenic glioma model was finally generated. Studies showed that there were lots of similarities between the making of glioma model and other tumor models. Some important DNA elements, such as the promoters and enhancers, modulated the transfected gene; the foreign genes fused with promoter and enhancer promoted a strong expression of target genes. The promoter or enhancer can be located in the upper stream or down stream of the genome, or it can be inserted in the non-transcribed DNA fragments (introns). There are constructed transgenic mice with astrocytoma, medulloblastoma, oligodendroglioma and multiforme glioblastoma.

#### **2.3.1 Genetic engineering mouse of astrocytoma**

Bachoo et al (Bachoo et al, 2002, Xiao et al, 2002) developed the mouse glioma model using retroviral carrying the active EGFR to infect astrocytes. They confirmed that injection of Ink4a/Arf<sup>-/-</sup> astrocytes expressing EGFR formed spheres into the animal's brain induced the formation of the high invasive astrocytoma. To simulate the RB mutations, Terry Van Dyke et al generated mouse astrocytoma model by introducing SV40T antigen (T121) which bind with RB and RB family members, P107 and P130 under the control of glial fibrillary acidic protein (GFAP) promoter. In 1995, Andrew Danks et al reported GFAP/SV40 TAg transgenic low-grade astrocytoma model. GFAP is the primary marker to identify the origin of the tumor, while SV40 TAg binds to P53 and inhibits its activity. After these two proteins were combined, the former regulated SV40 TAg and promoted its development of astrocytic tumors, while the latter suppressed the activity of wild-type p53 and promoted tumor development. As was shown in morphology and immunohistochemistry, astrocytes mainly were located in the subependymal zone in GFAP/SV40 TAg transgenic mice. Consistent with the characteristics of astrocytomas, some were located in the brain parenchyma or around neurons and blood vessel with GFAP expression. Chinese researcher Li HD reported that transfection myc andSV40TAg would promote cerebellum neuroblastoma and pancreatic cancer development.

#### **2.3.2 Genetic engineering mouse of oligodendroglioma**

Myelin basic protein (MBP) is an over-expressed protein during the formation of nerve myelin, which located in oligodendrocytes in the central nervous system or in Schwann cells in the peripheral nerves system. Oncogene neu was highly expressed in the majority of gliomas. In 1992, Hayes recombined Neu gene isolated from PSV2neu NT plasmid with MBP isolated from Cosmid (cos138) clones, and implanted it into C57BL / 6 × DBA / 2 F2 fertilized eggs. Of the 93 fertilized eggs injected with the recombined DNA, 14 of them had offspring. Four of the neonatal had brain tumors, which appeared in the underside of the

brain, the thalamus and the posterior fossa and compressed the brain stem. Under the light microscope, the brain tumors of three mice had the characteristic morphology of glioblastoma multiforme, and had extensive leptomeningeal invasion. Under the electron microscope, there were some characteristics of undifferentiated cells. To identify the origin of tumor cells, they used NF, GFAP, MBP and Leu 7 immunohistochemical staining. The results showed that there were GFAP, MBP and Leu 7 positive staining in relatively well-differentiated tumor cells, while in low differentiated tumor cells, less GFAP, MBP, and LEU 7 positive staining were found. None of the cells had NF positive staining. For the morphological analysis, Hayes's experiment did not successfully induce typical oligodendrocyte tumor, although the expression of MBP and Leu 7 indicated the presence of oligodendrocytes. The hybridization analysis was further used to clarify its origin, the results showed that a large number of neu gene presented in tumor cells, but not in normal brain cells. In addition, the results also showed that RNA level of myelin specific proteins MBP, PLP, MAG and CNP were 5-10 times higher in tumor cells than in the control group, which suggested that the myelin-forming cells was enriched in the tumor. There was no myelination protein Po found in peripheral nerve, indicating that tumor cells were derived from oligodendrocytes, but not from Schwann cells. Moreover, there was no NF detected in brain tumor cells, indicating that there was no neuron present in the tumors. Analyzed by the solid tumor markers, Hayes had established the transgenic mice of oligodendrocyte cell tumors.

### **2.3.3 Genetic engineering mouse of glioblastoma**

Injection of the combination of activated Ras and AKT into an Ntv-a transgenic mice induced glioblastoma tumor (Holland et al, 2000). When the DNA mixture was injected into Ntv-a mice with inactivate NK4a-ARF, the formation of the glioblastoma was accelerated. Ras and Akt work on the downstream signaling pathways of several growth factor receptors. In most of the glioblastoma multiforme tumors (GBMs), Ras and Akt are activated simultaneously. It has been proved that the abnormal expression of Ras can inhibit p53/RB pathway which induced the transformation of astrocytes into anaplastic astrocytoma cells that finally obtained GBMs characteristics after transfection AKT in the cells. These results confirmed the combined action of Ras and AKT in malignant glioma. Another GBMs model is generated in Nf1 and p53 double-silent mouse mated by the p53 knockout mice and the heterozygous of Nf1 and Cis (Reilly et al, 2000). Transplantation cells from such mice into other mice induced glioma or GBMs, which was caused by a depletion of a tumor suppressor gene instead of overexpression oncogene. Despite the molecular differences in previous GBMs models, they have something in common. Nf1 (nerve fiber) suppresses Ras activity, Nf1 knockout would lead to RAS activation. Nf1 alone is not sufficient to induce tumor formation, Nf1 combined with p53 deletion or lack of cell cycle regulation will induce tumor formation.

### **2.3.4 Genetic engineering mouse of medulloblastoma**

Generation of Ptch heterozygous mice is essential for the development of medulloblastoma transgenic mouse model. It is well known that Ptch receptor suppress proliferation through SHH / GLI signaling pathway. Inactivated Ptch receptor increases the risk for medulloblastoma, about 14% to 19% of the Ptch +/- mice develop medulloblastoma within 12 months, indicating that retained Ptch locus still function. When these mice were mated with the p53 deficient mice, the tumor incidence of their offspring increase to 95%, all



affected mice die within 12 weeks (Zurawel et al, 2000, Wetmore, 2001), suggesting that P53 plays an important role in this model.

The systemic experiment on the retroviral model and RCAS/tv-a indicated that SHH pathway was related to the formation of medulloblastoma. Under the guidance of ultrasound, utero injection of retroviral SHH directly into the cerebellum could induce medulloblastoma (Weiner et al, 2002). Fufts et al over-expressed SHH in newborn mouse cerebellum using RCAS / tv-a system, which induced the formation of medulloblastoma. c-Myc over-expression enhanced the induction. SHH's activities require the participation of Ptch. Inactivation of p53 and RB genes in neural progenitor cells in cerebellar granule cell layer developed brain tumor, indicating that RB family proteins may work on the tumorigenesis. P53 and RB conditional knockout mouse can produce a medulloblastoma, while single inactivated p53 or RB genes has no such effect. Some studies have shown (Tong et al, 2001) that adenosine diphosphate ribose polymerase (ADRP) is an early DNA damage response molecule. The mouse lack of ADP-ribose polymerase mate with the p53 gene deficient mice to generate ADRP and p53 double deletion mouse. Half of the mice have medulloblastoma located in the cerebellum.

### **3. Pathological features of transplantation tumor**

When human cancer tissue or cells are transplanted into animals, the micro-ecological environment has undergone tremendous changes. The cell morphology, molecular biology, host survival period, clinical symptoms of tumor are difficult to keep consistent with those of original tumors in many ways. Tumors developed with single cell suspension or ectopic transplantation have the interstitial and vascular components provided by the host (mouse), which is significantly different from clinical disease. For example, in human glioma subcutaneous xenografts model, the tumor weight up to 5-6 grams is not life-threatening to the 25 grams weighted nude mice. For the intracranial tumor, even when the inoculated tumors cover the entire cerebral hemisphere, or grow into the contralateral hemisphere, the cranial tumor-bearing mice are still alive (Figure 3). Therefore, to study the pathology of transplant tumor, we have to link them with clinical as closely as possible. There is no much references available, so we shared a few research data with all the readers here.

#### **3.1 Homogeneity**

Xenografts inoculated in nude mice can induce the transplant tumor having pathological features similar to the clinical specimens. Usually, the subcutaneous xenografts in nude mice are different from the original tumor. Brain glioblastoma multiforme tumor (GBM) transplanted subcutaneously in nude mice does not have the typical GBM features, but more like "fibrosarcoma". When the xenograft is inoculated in the brain, some invasive characteristics and molecular markers are consistent with the parental tumor. Our data proved that the parental tumor is non-invasive and overexpression of CEA, with acidic mucus secretion, the brain tumor metastases from lung cancer in nude mice can mimic these characteristics. While the highly invasive GBM with high expression of EGFR inoculated into nude mice brain showed highly invasive and EGFR over-expression characteristics. For morphology analysis, it is rarely identical. The morphology of implanted tumor has different characteristics depending on different locations, such as cerebral cortex, white matter, gray matter, ventricles and cerebellum.

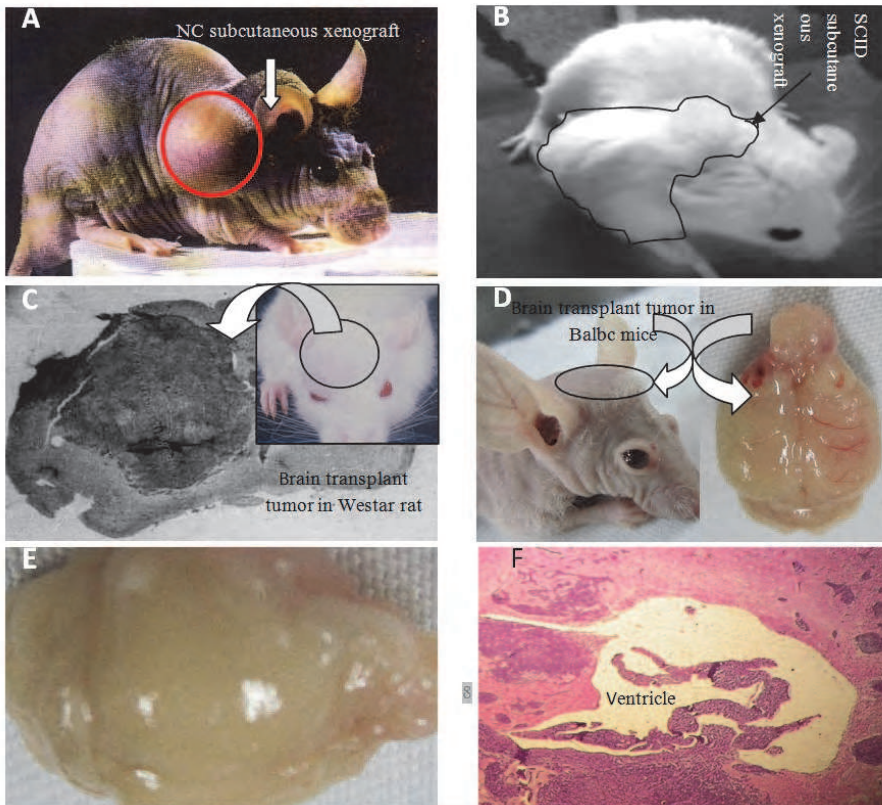


Fig. 3. NC glioma in nude mice, SCID mice transplanted subcutaneously, dexamethasone immunosuppressed Wistar rats and brain transplantation model in Balbc nude mice: the cells in subcutaneous space and in brain grow into large tumors due to unlimited proliferation. Intracranial skull uplifting can be observed in some mice (C, D black circle). Observed with cranial dissecting microscope or bear eye, the transplanted tumors almost covered the entire hemisphere (C arrow), or reached the olfactory lobe and the contralateral frontal lobe (D arrow). If spread to the choroid plexus, the tumor will cause hydrocephalus, both hemispheres are highly swollen (E) and ventricular expansion (F) will be noticed.

### 3.2 Mimesis

In the tissue repair process involving neural stem cells, the amplified daughter cells must have the same type of repair tissue to rebuild the function of cells, a phenomenon known as mimesis of neural stem cells, which had already been confirmed. We only focus on what the xenograft grows like and the tissue types of the parental tumor. When the glioma stem cells were implanted, we only checked whether the xenograft had neurons and glial cells features, rather than clarifying the subtype of the tissue. Our experiments have shown that stem cells derived from GBM grew diffusely, when it is located in the choroid plexus, it developed into choroid plexus carcinoma (Jun et al, 2010); when it settled in the ventricle wall, it developed into the ependymal neuroblastoma; when it settled in the brain surface

and spinal table, it developed into uniform small round cells (do not know the subtype). In short, stem cells derived from GBM implantation has no typical GBM characteristics, the type of transplant tumor highly depends on the location. We believe that glioma stem cells, like neural stem cells, have mimesis growing characteristics.

### 3.3 Remodeling

The tissue remodeling during the development is very common. In tumor development and progression, it is not conclusive whether there is remodeling between tumor and the host tissue cells, such as interstitial and vascular. Tumor vessel remodeling was first discovered in the malignant retina melanoma and the xenograft (Maniotis et al, 1999, Zhang et al, 2006). The vessels composed of tumor cells are called vessel memesis; while the vessels composed of tumor cells and endothelial cells are called mosaic blood vessels. We have made detailed observation on tumor vessel in malignant glioma (Yue et al, 2005). In human glioma transplanting into nude mice, the glioma stem cells migrated and proliferated around the host blood vessels. Besides relying on the host for the blood supplies, the tumor had spontaneous vascular memesis and mosaic blood vessels to provide nutrition for rapidly proliferating tumor. Moreover, some vascular wall cells had both host protein and tumor protein expression, indicating that in the process of tumor angiogenesis, the tumor stem cells as well as tumor-derived endothelial cells were directly involved.

There are several types of cancer stem cells and host cells remodeling. Besides the vascular remodeling, it is possible that the individual suspension glioma stem cells could remodel with host cells. There are following possibilities: (1) individual tumor cell relies on the host stromal cells for nutrition supply, therefore remodeling with the host vessel; (2) individual tumor cells fuse with host cells or other tumor cells to form multinucleated giant cells or aneuploid cells, which use the fusion cells to rebuild tumor blood vessels; (3) in very few cases, individual tumor cells, in particular disseminated tumor cells, transdifferentiate into vascular endothelial cells and build stem cell niche (Niche), which expand to form a distal distribution of tumor cells block. With the tumor progression, the host tissue gradually disappears among tumor mass.

### 3.4 Tumor imaging

The earlier imaging study about the animals bearing human brain tumor used single photon emission computed tomography (PET). Using tumor monoclonal antibody labeled with iodine radionuclides, the biological imaging of subcutaneous tumor had been collected by the  $\gamma$  camera and SPECT (single photon emission computer-aided tomography) machine in tumor-bearing mice, which may guide the diagnosis (Haubner et al, 2001, Herschman et al, 2003 Massoud & Gambhir, 2003.).  $^{18}\text{F}$  Radionuclide labeled glucose associated with tumor cell metabolism is also been used to trace tumor proliferation activity. Since most of the tumor-bearing animals are rats or mice, inoculated brain tumors are difficult to distinguish, both methods only work for the subcutaneous tumors. With the improvement in scientific technology, foreign researchers have used animal-specific high magnetic field MR (3-17.6T) on rats or mice for conducting imaging studies of intracranial tumors (Beck et al, 2002, Lewis et al, 2002, Pirko et al, 2005). Chinese researchers are still using ordinary field MR (1.5T) for imaging studies of rat intracranial tumors. Recently, we used rats and mice specific small coil 1.5T MR machine for imaging studies of orthotopic glioma in nude mice. We got some satisfactory results, which can be used for monitoring tumor and adjacent structures and

calculating tumor volume. But it will take a long imaging time and can not mimic the patient pictures.

Tumor vessel imaging is part of the tumor imaging. Percutaneous transcatheter and intravascular injection of contrast agents, and digital subtraction angiography (DSA) are used to get tumor microvascular data. However, the transplanted tumors mostly use small rodent animals, on which the DSA technology can not be applied. The vascular endothelial cell marker, such as CD34 + was used to calculate the vascular density and evaluate the number of tumor blood vessels, and the therapeutic effect of anti-tumor angiogenesis drugs. However, this method can not be used to evaluate the transporting function of these vessels. The cancer stem cell self-generated blood vessels (vasculogenesis) different from the host vascular endothelial cells formed blood vessels (angiogenesis). We established the activated carbon granular heart chamber perfusion method, which confirmed that various types of tumor vessels are involved in tumor-bearing animal systemic circulation (Figure 4) (Dong et al, 2010). The detailed procedures are as following: (1) producing activated carbon suspension: activated carbon particles were ground into powder and added to the PBS to make the suspension. Then the mixture was filtered through 40-micron net (U.S. BD Company). After rested for 1 min, the carbon particle was sucked by a flat cut, polished needle; (2) infusing activated carbon suspension: the tumor-bearing mice were anesthetized by 10% chloral hydrate. The syringe was pierced through the left ventricle of mice, while a small hole was cut in the right atrial appendage of the heart to facilitate the replacement of circulating blood. After 2-3 ml of carbon suspension was infused, the tumor tissue was removed, fixed in 4% paraformaldehyde and embedded in paraffin; (3) analyzing the perfusion results: active carbon particle suspension in left ventricular cavity went into host circulation and the tumor blood circulation. The active carbon particle distribution was related to the blood density. There were small carbon particles in host large vessels and new bleeding necrotic area, and integrated condensate active carbon particles in the medium-sized blood vessels, aggregated carbon particles in the tumor microvascular. It is worthy to mention that the active carbon particles would leak out of the lumen because the carbon particle is less than 40 microns in diameter, smaller than the red blood cells. It is interesting that the carbon particles were present in the marker of highly malignant tumor "false daisy group" and "tumor necrosis". For the former one, few carbon particles were observed in the central lumen, while in the latter structure, large piece of carbon particles were scattered in the gap of sparse distributed tumor cells. Since there is no red blood cells or other tangible materials found, suggesting that this change existed after tumor necrosis. The present carbon particles indicated that there was nutrition supply, suggesting that necrotic tumors tissue might have a micro-environment promoting "self-healing" process.

Imaging of fluorescent protein tracer is a newly developed technology. We had inoculated human glioma stem cells SU3 transfected with red fluorescent protein (RFP) into BALBc nude mice brains to trace its location (Fig. 5). Hoffman (Hoffman, 2002) had inoculated U87 glioma cells transfected with RFP into the brains of nude mice expressing green fluorescent protein (GFP). Farin et al (Farin et al, 2006) had injected C6 glioma cells labeled with eGFP and DsRed-2 into the forebrains of neonatal rats, and used fluorescence imaging to observe dynamic tumor growth *in vivo*. Although clarity of the imaging, precision of the display of tumor size, location and depth are still not satisfying, this method will be widely used with the development of technology. In this platform, the outstanding finding was that glioma cells invaded brain tissue along the cavity of blood vessels. Tumor cell intruded into the endothelial cells and the pseudo foot of astrocytes, not the vascular cavity. In addition, glioma cells jumped forward sometimes slowly and sometimes rapidly, with the maximum

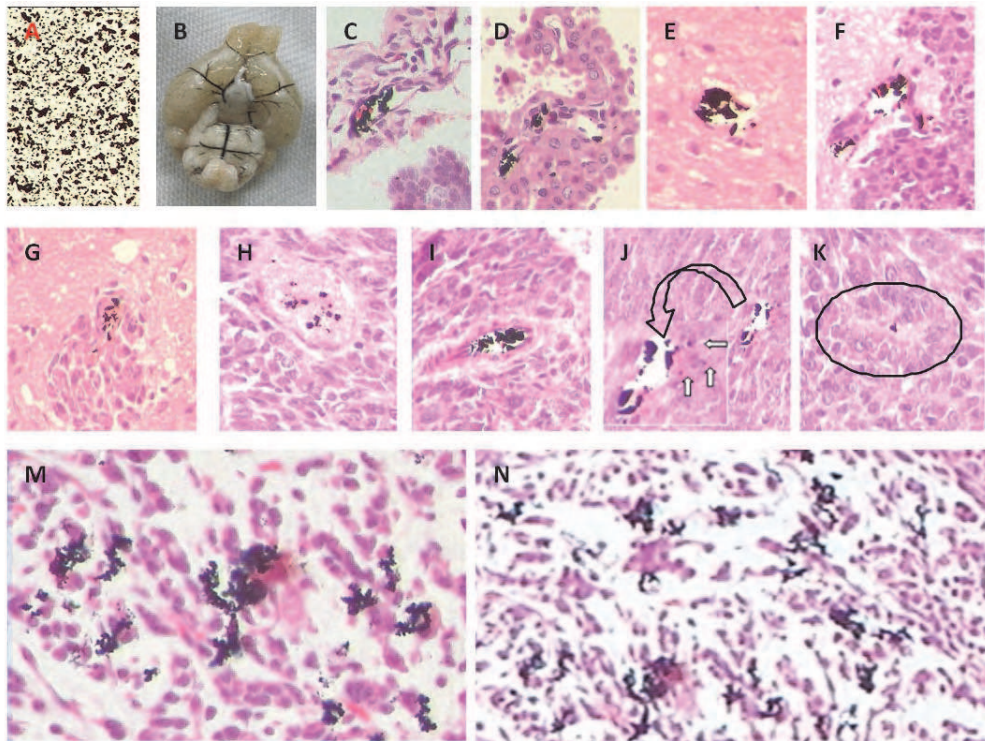


Fig. 4. Carbon particles ventricular perfusion in tumor-bearing mice, to trace blood flow of new Integrated tumor vessels. When carbon particles are perfused in tumor-bearing mice hearts, the blood flow of new tumor vessels is showed as following: A: suspension of carbon particles by light microscopy; B: the ventral surface profile of brain tumor, showing the basilar artery and Willis ring filled with carbon particles; C-K: the HE sections, showing that the carbon particles go into the following vascular tissue: subarachnoid space (C); choroid plexus (D); normal brain tissue (E); tumor margin (F); tumor foci (G); massive tumor tissue, including some endothelial-dependent blood vessels (H, host blood vessels) and some blood vessels formed by tumor cells (I, vascular mimesis); carbon particles leaking from tumor-origin blood vessels (J, arrow in magnified square); carbon particles in the central vessel of false daisy (K, circle); besides, carbon particles existing outside of vessels in the tumor necrosis, in acute necrotizing period, carbon particles and floating red blood cells coexist (M); after the acute period, carbon particles exist in absorbed lesions (N), indicating that there are blood supply even in the repair period of tumor necrosis.

speed over 100um per second; the migrated cells would go through proliferation and division. Cells divided at or near the vascular bifurcation. This was the first time the comprehensive kinetic data about glioma cell infiltration *in vivo* was recorded, which indicated that the proliferation and migration of glioma cells related closely with host vascular system. Considering from the reconstruction of tumor tissue, host's own tissues and cells also play an important role and provide nutrition for the growth of tumor. Yang (Yang et al, 2004) and we had inoculated human glioma stem cells non-transfected and



transfected with RFP into nude mice brains. The former model (Jun et al, 2010) had proved that cancer stem cells involved in tumor blood vessel formation and fusion with host cells; In the latter model, host cells were proliferating actively around the tumor, and active host cells cultured *in vitro* have immortalized features (Figure 6).

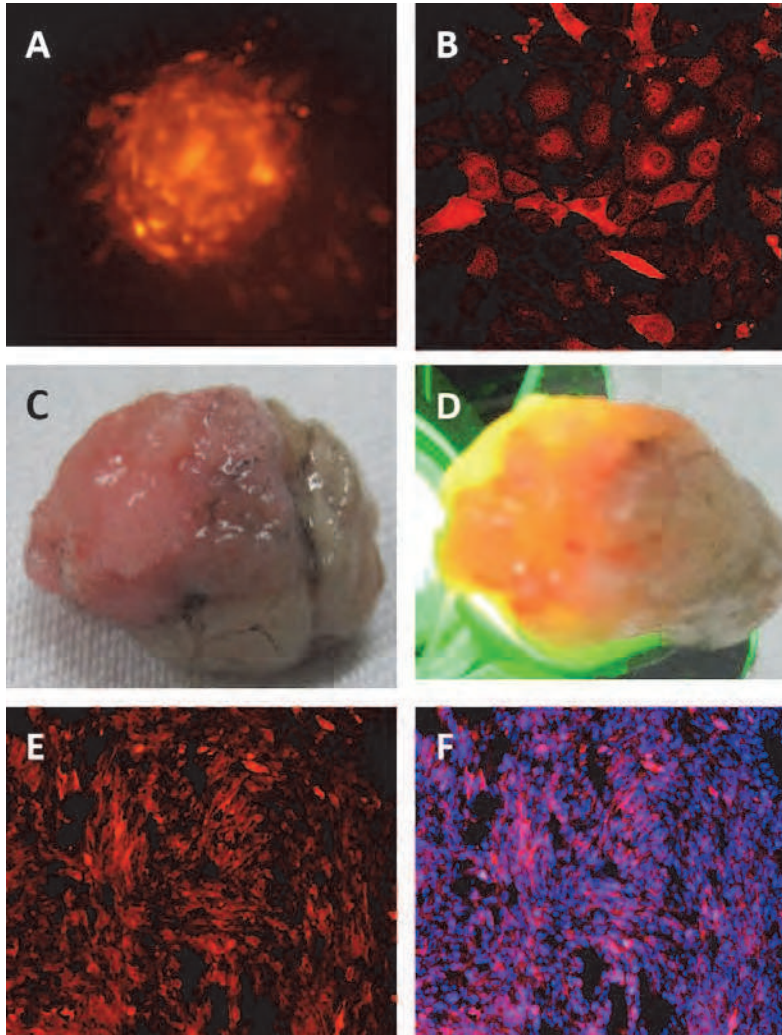


Fig. 5. Tracing human glioma stem cells SU3 and orthotopic transplantation tumor in Balb/c nude mice with red fluorescent protein (RFP) mice: A and B: the SU3 with RFP transfection cultured with growth factors or serum under optical phase contrast microscope; C and D: the brain tumor observed in natural light and excitation light, red swollen tumor in the front of the brain; E and F: the tumor tissue sections under confocal microscope, the red tumor cells (E) and the nuclear staining (F) crowdedly arranged, suggesting a high degree of malignancy.

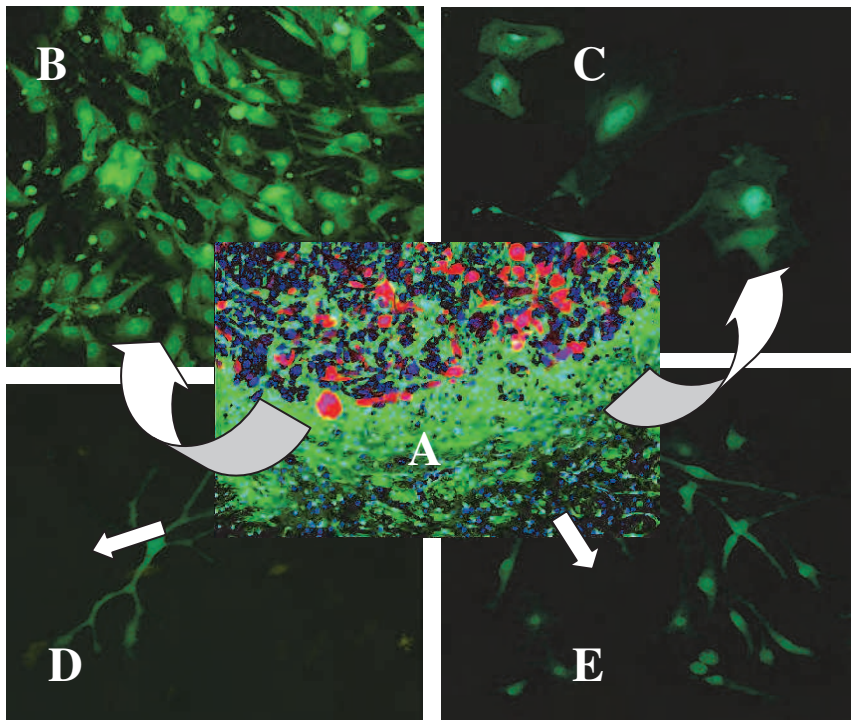


Fig. 6. The image of RFP / GFP brain tumor biopsy under fluorescence scanning confocal microscopy. RFP/GFP brain tumor biopsy under fluorescence scanning confocal microscope (A), human glioma stem cells (SU3) transfected with RFP is red, host (NC/C57BL/6J-GFP nude mice transfected with GFP) tissues and cells is green, the nucleus is blue. There are host tissues in tumor interstitial and around the active zone, where the high proliferative host cells crowded together. The morphology are diverse among the cell suspension cultured from the tissue taken from the active zone under the the fluorecence microscope. Some of them are pleomorphic tumor cells, with several features: star shape, highly proliferative, rapidly covering the culture bottom, monoclonal passaged (B); others are macrophages with putiplnucleus (C); neurons (D) and fiberblast (E).

The establishment of fluorescent protein transfected tumor cells and transgenic mouse tumor model has made a significant contribution to the medical research. There are lots of application followed by pitfalls: (1) although fluorescent protein is less toxic to cells, it has cytotoxic effects to liver, heart and nervous stem cells (Huang et al, 2000); (2) tumor cells expressing fluorescent protein may be engulfed by phagocytic cells invaded into the tumor area, it is impossible to distinguish them using current technology, which may lead to a false judgment of the experimental results; (3) limited by the current technology, the method can not fully meet the needs of interpreting fluorescent tracer image. However, we have every reason to believe that with the introduction of new imaging technologies, fluorescent tractor will make greater contribution to cancer research. For example, neural stem cells and cancer stem cells transfected with different colors of fluorescent protein are likely to help distinguish the origin of tumor cells; in the tumor micro-ecology research, the improved

technology will help to dissect the components of cancer stem cell niche; in tumor evolution, it is known that anaplastic cell is the basis for malignant tumor, tumor cells and host cells transfected with different colors will help to elucidate whether cell fusion could contribute to the malignant progression. Finally, during the reconstruction process of transplant tumor, the donor and receptor with different fluorescent proteins are expected to be used to record their roles non-invasively.

#### 4. Acknowledgments

This chapter is supported by National Basic Research Program of China (973 program, 2010CB529403), National Natural Science Foundation of China (No. 30872654, 81071766), Natural Science Foundation of Jiangsu Province (NO. BK2008173, 2010227) and the Foundation of Suzhou YJ S0950.

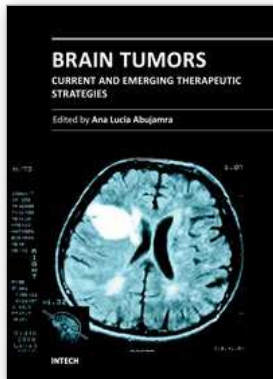
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## **Brain Tumors - Current and Emerging Therapeutic Strategies**

Edited by Dr. Ana Lucia Abujamra

ISBN 978-953-307-588-4

Hard cover, 422 pages

**Publisher** InTech

**Published online** 23, August, 2011

**Published in print edition** August, 2011

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### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Chen Hua, Dong Jun and Huang Qiang (2011). Xenograft Model of Human Brain Tumor, Brain Tumors - Current and Emerging Therapeutic Strategies, Dr. Ana Lucia Abujamra (Ed.), ISBN: 978-953-307-588-4, InTech, Available from: <http://www.intechopen.com/books/brain-tumors-current-and-emerging-therapeutic-strategies/xenograft-model-of-human-brain-tumor>

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