DEVELOPMENT OF NEW HUMAN STEM CELL-DERIVED CELLULAR VEHICLES FOR GLIOMA GENE THERAPY

ZHAO YING

NATIONAL UNIVERSITY OF SINGAPORE

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ZHAO YING

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SUMMARY

Malignant glioma remains one of the most lethal forms of cancer in humans. However, current therapy for glioma rarely achieves long-term tumor control. Stem cell–based gene therapy is a promising new strategy for the treatment of glioma. Neural stem cells are highly efficacious in targeting brain tumors and show a specific affinity for invading glioma cells. Genetically engineered neural stem cells expressing therapeutic genes can inhibit the growth of glioma, facilitate elimination of tumor cells, and repair damaged brain tissue. As such, neural stem cells may be effective delivery vehicles for gene therapy to malignant neoplasms in the brain. However, the mechanism of glioma-tropic behavior in neural stem cells is not well understood. Furthermore, there are significant ethical issues limiting the use of stem cells of fetal origin. This study aimed to discover new regulators that might enhance cell migration toward gliomas and sought to develop alternative, large-scale sources of neural stem cells for use in gene therapy for glioma.

In this study, we identified and characterized a novel cell motility modulator, TMEM18. Overexpression of TMEM18 was observed to provide neural stem cells and neural precursors an increased capacity to migrate toward glioblastoma cells, both *in vitro* and in the rat brain. Functional inactivation of the *TMEM18* gene resulted in almost complete loss of migration activity in these cells, demonstrating that TMEM18 is a novel cell-migration modulator.

Overexpression of this protein could be used favorably in neural stem cell–based therapy for glioma.

A population of human glioma-tropic precursor cells, NT2.RA2 migrating cells, was then derived from retinoid acid (RA)–treated neural precursor NT2 cells. After systemic administration in nude mice, the NT2.RA2 migrating cells targeted intracranially and subcutaneously implanted U87 gliomas. When genetic engineered to express the suicide gene HSVtk, NT2.RA2 migrating cells showed significant antitumor effects and prolonged the animals' survival. Thus, we had successfully derived glioma tropic precursor cells from NT2 cells and used them as efficient delivery vectors in gene therapy for glioma.

Finally, this study demonstrated, for the first time, that human embryonic stem cells can provide a potentially unlimited source for glioma gene therapy. Using a novel monolayer culture condition, we successfully derived long-term proliferating neural stem cells from HES1 and HES3 human embryonic stem cell lines. The embryonic stem cell-derived neural stem cells showed strong glioma-specific tropic behavior in Boyden migration assays. When carrying the suicide gene HSVtk, these cells possess resistance to phospho-GCV, and demonstrated strong antitumor effects *in vitro*.

This work may improve brain tumor gene therapy and provide unlimited, clinically viable cell sources for use as vehicles for gene delivery. We hope

that this thesis will lead to improvements in glioma therapy and help prolong the survival of patients with malignant glioblastomas.

LIST OF PUBLICATIONS

Jaana Jurvansuu*****, **Ying Zhao***, Doreen S.Y. Leung, Jerome Boulaire, Yuan Hong Yu, Sohail Ahmed, and Shu Wang. Transmembrane Protein 18 Enhances the Tropism of Neural Stem Cells for Glioma Cells. *Cancer Research.* 68: 4614-4622. 2008. (***** co-first authors)

Ying Zhao, Doreen S.Y. Leung, Shu Wang. Glioma Tropic Stem Cells Derived from Human NT2 Cells. *SBE's 3rd International Conference on Bioengineering and Nanotechnology.* Singapore. 2007.

Ying Zhao, Doreen S.Y. Leung, Shu Wang. Targeted Suicide Gene Therapy of Malignant Glioma Using Glioma Tropic Precursor Cells Derived from NT2. In preparation.

Jieming Zeng, Juan Du, **Ying Zhao**, N Palanisamy and Shu Wang. Baculoviral Vector-mediated Transient and Stable Transgene Expression in Human Embryonic Stem Cells. *Stem Cells.* 25: 1055-1061. 2007

The studies presented in this thesis are based on the research work in the above publications and manuscript.

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ABBREVIATIONS

GBM	Gliomblastoma multiforme
BBB	Blood-brain barrier
CNS	Central nervous system
HSV-1	Herpes simplex virus type 1
MoMLV	Moloney murine lerkemia virus
PEI	Polyethylenimie
ESC	Embryonic stem cell
ASC	Adult stem cell
HSC	Hematopoitic stem cell
NSC	Neural stem cell
MSC	Mesenchymal stem cell
EGF	Epidermal growth factor
bFGF	Basic fibroblast growth factor
EB	Embryoid body
SDF-1	Stromal cell-derived growth factor
CXCR4	CXC chemokine receptor 4
VEGF	Vascular endothelial growth factor
HSVtk	Herpes simplex virus-thymidine kinase
GCV	Ganciclovir
CD	Cytosine deaminase
CE	Carboxylesterase

IL	Interleukin
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
IFN-β	Interferon-β
NPC	Neural precursor cell
TMEM18	Transmemebrane protein 18
NT2	NTera2/D1
RA	Retinoic acid
ATCC	Ameracon type culture collection
NIH	National Institute of Health
mEF	Mouse embryonic fibroblast
RT-PCR	Reverse transcription PCR
NSL	Nuclear localization signal
GFP	Green fluorescence protein
GFAP	Glial fibrillary acidic protein

CHAPTER 1

INTRODUCTION

1.1 Brain tumors

Malignant brain tumors are one of the most devastating forms of human cancers. With an incidence of just 1 in 10,000 in Western countries, they are responsible for about 2% of all deaths (Counsell and Grant, 1998; Pobereskin and Chadduck, 2000). In adults, one-half of brain malignancies are primary and the rest metastatic (Annegers et al, 1981). Brain tumors are classified on an ascending scale of malignancy from I to IV according to cell type (Louis et al, 2007). Grade IV gliomas are most common in the elderly, while medulloblastomas have the highest incidence in children.

1.1.1 Gliomas

Gliomas can arise from either astrocytes or oligodendrocytes (Berger, 1998). About 50% of primary neoplasms are gliomas and 50% of gliomas are the most malignant type, glioblastoma (Kleihues and Sobin, 2000). The incidence of malignant gliomas seems to be increasing, especially in the elderly (Hess et al, 2004). Malignant glioma remains one of the most lethal forms of cancer in humans, with average survival of less than 1 year. Grade IV glioma, known as glioblastoma multiforme (GBM), is the most malignant. A recent population-based study showed that the survival of patients with glioblastoma multiforme was 42.4% at 6 months, 17.7% at 1 year, and 3.3% at 2 years (Ohgaki et al, 2004).

Highly aggressive gliomas develop either *de novo* (primary) or progress from lower grade tumors through mutation (secondary). The origin of primary brain tumors is not clear. Recent studies of cancer stem cells show that brain tumor stem cells are crucial for the initiation and maintenance of gliomas. A population of glioblastoma stem cells expressing the neural stem cell (NSC) marker CD133 was first isolated from brain tumors (Singh et al, 2004). Like NSCs, glioblastoma stem cells possess the fundamental stem cell properties of self-renewal and multipotency. The most important feature of CD133+ cells is that they will generate secondary tumors when transplanted into the striatum of adult immunodeficient mice, demonstrating their self-renewal *in vivo* (Galli et al, 2004). Studies of the origin of brain cancer stem cells reveal that they arise from the malignant transformation of normal somatic stem cells or of more mature cells within the high-proliferation zone, such as the subventricular zone (Vescovi et al, 2006).

Glioma cells can infiltrate into normal brain tissue and migrate long distances. It has been reported that gliomas infiltrate and migrate along perivascular, perineuronal, and subpial spaces, as well as white matter such as the corpus callosum (Holland, 2000). The highly invasive nature of glioma makes it impossible to surgically remove the entire tumor mass. Remnants cause tumor recurrence and lead to patient mortality.

1.1.2 Glioma therapy: current status and challenge

Current therapy for intracranial glioma is most commonly surgical resection with adjuvant radio- or chemotherapy. Despite dramatic advances in neurosurgery, radiotherapy, and chemotherapy in recent decades, the median survival of patients with malignant glioma remains unchanged, at about 12 months.

After neurosurgical resection, the survival of patients with glioma may be prolonged by up to 6 months (Shand et al, 1999). Recent advances in surgical techniques have improved the treatment of glioma. Neurosurgeons can now locate and characterize lesions using new imaging technologies, such as high-resolution magnetic resonance imaging (MRI), MR spectroscopy, positron emission tomography (PET) scans, and diffusion and perfusion imaging (Nelson and Cha, 2003). Precise and aggressive surgical tumor resection can be achieved using combined frameless stereotaxis and intraoperative MRI translated imaging (Oh and Black, 2005). However, the lack of a defined tumor edge makes resection difficult. In addition, brain tumors may invade normal brain tissue and may form in critical areas.

Radiotherapy may be used as follow-up treatment to kill residual tumor cells after surgical resection. It may also be employed when the tumor is in an area that renders it inoperable. Normal brain tissue can tolerate up to 60 Gy of radiation, which is below the threshold required to kill glioma cells. Several

new technologies, including hyperfractionated radiation therapy, stereotactic radiosurgery or radiotherapy, interstitial radiotherapy, and boron neutron capture therapy have been used to enhance the efficiency of radiotherapy while minimizing side effects. Although these technologies reduce the radiation to some degree within normal brain tissue, they do not significantly improve therapeutic efficiency.

Chemotherapy may be used at initiation of glioma therapy or following surgery and/or radiotherapy. Chemotherapy of brain tumors is not curative; its goals are to control tumor growth and maintain the patient's quality of life for as long as possible (Castro et al, 2003). The most commonly used chemotherapy drugs are nitrosoureas (BCNU, CCNU); platinum-based drugs (cisplatin, cisplatinum, carboplatin); temozolomide; procarbazine; and naturally occurring compounds such as taxol (Burton and Prados, 2000). Glioblastoma tends to be more resistant than other types of brain tumors. Use of multiple types of antitumor drugs sometimes overcomes this chemoresistance, but cells within the tumor mass have different sensitivities to the drugs. Cells with lower sensitivity can produce resistant clones, which may then develop secondary tumors.The blood - brain barrier (BBB), may also cause chemotherapy to fail. The BBB is a physical barrier separating the brain from the blood and prevents most hydrophilic substances and large hydrophobic molecules from reaching the tumor site through passive diffusion. Efflux pumps presenting in BBB, such as P-glycoproteins, organic anion

transporters, and multidrug resistance–associated proteins may also pump foreign molecules out of the brain.

Current glioma therapy is often not curative and rarely achieves long-term tumor control. Thus there is a great need for novel therapies.

1.2 Glioma gene therapy

Because gliomas consist of localized dividing cells and seldom metastasize outside the central nervous system (CNS), gene therapy is a promising new treatment. It would allow vector delivery directly to the tumor site, reducing the risk of systemic side effects (Immonen et al, 2004). The goal of glioma gene therapy is to achieve therapeutic-level transgene expression at the tumor site while minimizing damage to the surrounding normal brain tissue. Although glioma gene therapy has produced encouraging results in animal models, clinical trials have not yet achieved considerable therapeutic effect because of low gene transduction in patients. Viral, chemical, and cellular vectors are being studied as vehicles for gene delivery.

1.2.1 Viral vectors

Viral vectors are the most effective *in vivo* gene delivery reagents and have been well studied in clinical trials. Adenovirus, retrovirus, and herpes simplex virus type 1 (HSV-1) are the most commonly used viral vectors in glioma gene therapy.

Adenovirus is a nonenveloped particle with a double-stranded DNA genome of 36 kb (Horne et al, 1975). Adenovirus vectors are used in replication-deficient and replicating forms (Chiocca et al, 2003). After deletions in the early regions of the adenoviral genome, adenovirus vectors become replication deficient. In glioma gene therapy, replication-deficient adenovirus can transduce dividing and nondividing cells efficiently without risk of insertion mutagenesis, and its safety has been proven in a number of clinical trials (Immonen et al, 2004; Trask et al, 2000). Adenovirus has high antigenicity *in vivo*, especially in early-generation adenoviruses. The immune responses activated by the adenovirus virions may have provided additional antitumor effects in glioma treatment (Danthinne and Imperiale, 2000; Kay et al, 2001; Sandmair et al, 2000). Replicating viruses are oncolytic, selectively lysing, dividing tumor cells, and thus spread throughout the tumor (Chiocca, 2002). There are several ways to engineer replicating oncolytic adenovirus to achieve tumor selectivity. Replicating adenovirus mutated in E1A specifically lyses retinoblastoma-defective glioma cells (Fueyo et al, 2000). The ONYX-015 vector mutated in E1B restricts virus replication to p53-deficient tumor cells, and its tumor-specific lysis has been enhanced in clinical trials when combined with chemotherapy (Bischoff et al, 1996; Heise et al, 1997). An adenovirus mutated in both E1A and E1B showed a potent antitumor effect in intracranial glioma xenografts, with increased tumor selectivity (Gomez-Manzano et al, 2004).

Retrovirus vectors are enveloped RNA viruses derived primarily from Moloney murine leukemia virus (MoMLV), with a transgene capacity of up to 8.5 kb. During transduction, the virus RNA is reverse transcripted to double-stranded DNA, which is then transported to the nucleus and randomly integrate into the host cell genome. Retrovirus vectors are usually used in replication-deficient form, which is rendered by deleting the genes gag, pol, and env. Retrovirus vectors can be delivered directly by intratumor injection. A more efficient method is to graft the engineered vector-producing cells intratumorally to produce virus *in situ* (Rainov and Kramm, 2003). However, the application of retrovirus vectors in glioma gene therapy is limited by the vectors' inability to infect nondividing cells and by low transduction efficiency *in vivo* (Rainov and Ren, 2003; Vile and Russell, 1995). To improve transduction efficiency, replication-defective HSV-1 or adenovirus has been used to deliver retrovirus packaging sequence and transgene directly to the tumor site, transforming the tumor cells to vector-producing cells and increasing transgene expression (Hampl et al, 2003).

HSV-1 is an enveloped virus carrying a double-stranded DNA of 152 kb. HSV may prove particularly useful in the treatment of gliomas located in the CNS because of viruses' known tropism for nervous tissue (Barnett et al, 1999; Lilley et al, 2001). Similar to adenovirus, replicating and replication-deficient HSV-1 vectors have been developed for use in glioma gene therapy.

Replicative HSV-1 vectors mutated in neurovirulence or ribonucleotide reductase genes showed tumor-specific replication and lysis in early-phase clinical trials (Shah et al, 2003; Varghese and Rabkin, 2002).

1.2.2 Chemical vectors

In clinical trials, chemical vectors have shown lower transfection efficiency *in vivo* with fewer safety concerns than with viral vectors. Naked DNA, liposome, and DNA/polymer complex are currently being studied.

Naked DNA does not cause an immune response against the carriers. Physical modification, including calcium phosphate precipitation, DEAE-dextran/chloroquine permeabilization, heat shock, and intracellular microinjection is required for naked DNA to enter host cells (Castro et al, 2003). Unfortunately, most of these techniques are restricted to *in vitro* gene delivery and transfection efficiency is quite poor. Naked plasmid DNA has been used only occasionally in glioma gene therapy (Barnett et al, 2004).

Liposomes are highly successful in transfecting cell lines; several clinical trials have used liposomes in glioma gene therapy. One brain tumor trial in humans used cationic liposomes to deliver therapeutic genes (Yoshida et al, 2004). Immunoliposomes conjugated with monoclonal antibodies have also been reported to target glioma cells (Zhang et al, 2004). However, liposomes are limited by ineffective delivery *in vivo*.

A series of polymers has been developed to facilitate gene delivery. Among them, the polycationic polymer polyethylenimine (PEI) has shown high transfection efficiency *in vitro* and *in vivo.* PEI-siRNA complex was reported to exert antitumor effects in an animal glioma model (Grzelinski et al, 2006).

1.2.3 Cellular vectors

In gene therapy, the transgene could be delivered directly by viral or chemical vectors (*in vivo* gene transfer) or delivered to donor cells that are later transplanted in the patient (*ex vivo* gene transfer). *Ex vivo* gene therapy allows the characterization of transfected cells before grafting and the selection of transgene-expressing cells. Both somatic and stem cells are used as donor cells (Tinsley and Eriksson, 2004). In glioma gene therapy, stem cell vectors provide more advantages, such as homing patholigies and damage-repairing capacities. In the following sections, we discuss stem cell–based glioma gene therapy in detail.

1.3 Stem cell–based glioma gene therapy

Glioma gene therapy clinical trials over the past 10 years have tested adenovirus, retrovirus, HSV-1, and liposome vectors. The results of most of these clinical studies have been poor, and transfection efficiency of these vectors was low *in vivo*. These poor results were due to the inability to kill tumor cells *in situ* and the limited distribution of transgene and vectors within

the tumor mass. Stem cells are recently merged gene-delivery vectors for glioma gene therapy and could resolve this difficulty.

1.3.1 Stem cells: embryonic and adult

Stem cells have two important features distinguishing them from other cell types. One is self-renewal, meaning that these cells renew themselves for long periods by cell division. Second, under certain conditions, stem cells can give rise to one or more mature cell types. Stem cells are composed mainly of embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are primitive (undifferentiated) cells from embryos that have the potential to become a wide variety of specialized cell types; ASCs are undifferentiated cells found in a differentiated tissue that can renew itself and (with certain limitations) differentiate to yield all the specialized cell types of the tissue from which they originated (*Stem Cells: Scientific Progress and Future Research Directions*, NIH, 2001).

1.3.1.1 Embryonic stem cells (ESCs)

Mouse ESCs were first isolated in 1981 by two independent groups (Evans and Kaufman, 1981; Martin, 1981). Extensive studies of mouse ESCs have broadened our understanding of these cells' early development and differentiation pathways. The later successful isolation of human ESCs encouraged today's tremendous interest in the potential therapeutic applications of stem cells. In 1998, Thomson first isolated human ESCs from

the inner cell mass of the blastocyst stage (100-200 cells) of embryos generated by *in vitro* fertilization (Thomson et al, 1998). Other methods were developed to derive human ESCs from the late morula stage (30-40 cells) (Strelchenko et al, 2004), arrest embryos (16-24 cells incapable of further development) (Zhang et al, 2006), and single blastomeres isolated from eight-cell embryos (Klimanskaya et al, 2006).

ESCs can proliferate without limit and differentiate into derivatives of all three germ layers (ectoderm, mesoderm, and endoderm). Human ESCs provide an unlimited source of normal human differentiated cells, offering great potential applications in basic developmental biology studies, drug screening, degenerative diseases, and gene therapy. For example, studying the pathways involved in the development of human embryos would yield a better understanding of fetal development that could then be used in the prevention and treatment of birth defects. Second, the pluripotency of human ESCs allows the establishment of various new cell-culture models for drug screening and toxicity testing. Third, using well-defined protocols, human ESCs could be directed toward specific cell types; for example, insulin-producing cells and neurons. These cells could then be used in transplantation therapies for degenerative diseases such as diabetes and Parkinson's disease. Finally, human ESCs provide an unlimited supply of cellular vectors for novel *ex vivo* gene therapy. After genetic modification by

virus or liposome, cellular vectors derived from human ESCs could be transplanted into the patient to deliver the therapeutic gene.

1.3.1.2 Adult stem cells (ASCs)

ASCs were originally isolated from adult tissue. Their role is to maintain mature cell types in steady-state numbers and replace cells that have died due to injury or disease. Unlike ESCs, ASC proliferation is limited; ASCs can differentiate only into the cell types specific to their originating tissue. However, the therapeutic applications of ASCs are much better studied in clinics. Hematopoietic stem cells (HSCs) are the best characterized and understood ASCs in therapeutic application. Syngeneic and allogeneic HSC transplantations can be used to replace depleted hematopoietic systems and induce immune tolerance in patients with severe aplastic anemias, fatal leukemias, and other hematological malignancies (Denham et al, 2005). HSCs (Aiuti et al, 2002), mesenchymal stem cells (MSCs) (Nakamura et al, 2004), and neural stem cells (NSCs) (Aboody et al, 2000) have demonstrated utility in animal models undergoing gene therapy.

1.3.1.2.1 Embryonic stem cells versus adult stem cells

The differences between ESCs and ASCs have been reviewed by Cheng (2008) (Table 1.1).

Table 1.1 Fundamental differences between ESCs and ASCs (Cheng, 2008).

	ESCs	ASCs
Origin	Blastocyst	Developed tissue
Proliferation *in vitro*	Indefinite	Limited
Differentiation spectrum	All tissue types	Limited
Homing ability	No	Yes
Reconstitution efficiency	Low	High
Tumorigenesis	Teratoma	No or rare
Availability	Restricted (human)	Less restricted
Ethical issues	Severe (human subjects or human-xeno models)	Fewer
Clinical proof	No	Yes (HSC)
Most challenging technique in therapeutics	Efficient induction of specific tissue types without tumorigenesis	*In vitro* expansion without loss of physiological properties

ESC research is still in an experimental stage, but ASCs have become therapeutically usable. In the future, the therapeutic choice between ESCs and ASCs will depend on the specific tissue types required by the diseases.

1.3.2 Neural stem cells (NSCs)

NSCs are multipotent cells with the ability to self-renew and generate mature cells of all three fundamental neural lineages (neurons, astrocytes, and oligodendrocytes) throughout development, as well as to reconstitute those cell types in damaged regions of the CNS (Parker et al, 2005).

NSCs like “proliferating neurons” were first identified in the adult rat brain in 1965 (Altman and Das, 1965). NSCs were later isolated from the embryonic and adult CNS. In embryos, NSCs are isolated from the ganglionic eminence, whereas in adults NSCs are isolated from the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (Gage, 2000). After the *in vivo* identification of NSCs, several procedures were developed to propagate NSCs *in vitro*. Commonly, rodent and human NSCs isolated from fetal and adult brains are expanded as neurospheres from single cells in a serum-free medium with both epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Piper et al, 2001; Tropepe et al, 1999). When EGF and bFGF are withdrawn, NSCs give rise to neurons, astrocytes, and oligodendrocytes.

NSCs can also be derived from ESCs. It seems that neuronal fate is most favored by ESCs when there is no other instructive cue (Smukler et al, 2006). Many NSCs and other specific neural cells, such as dopamine neurons and motor neurons, have been differentiated from mouse and human ESCs. A

variety of methods have been developed to induce NSCs from human ESCs, but most of these methods have used the formation of neurospheres or embryoid bodies (EBs). NSCs could be generated by overgrowth of human ESCs to a higher cell density (Reubinoff et al, 2001). After prolonged culture of human ESCs, without changing feeder cells for 3 to 4 weeks, NSC marker-positive cells are mechanically isolated and put into the serum-free medium to form neurospheres. EBs may also be formed to induce neural differentiation of human ESCs (Carpenter et al, 2001; Zhang et al, 2001). The EBs are subsequently seeded onto an appropriate substrate in a defined medium containing mitogens to further select NSC population. However, in cell culture, the proliferation of NSCs derived by neurospheres and EBs is limited, and the difficulty of handling cell aggregations limits large-scale preparation. In addition to neurosphere and EB formation, directed differentiation of ESCs to NSCs has been achieved by coculture with mouse PA6 stromal cells (Song et al, 2007). However, exposure to animal cells is a safety concern when considering therapeutic applications.Recently, Smith and colleagues showed that simple plating of mouse ESCs and human embryo cells in monolayer culture could successfully develop NSCs (Conti et al, 2005; Ying et al, 2003). This novel and straightforward method makes bulk preparation of NSCs from ESCs possible.

NSCs represent a renewable source for transplantation therapies in neuronal disorders. After transplantation, exogenous NSCs integrate seamlessly in

large numbers into the surrounding host brain tissues and differentiate into three neural lineages, making them attractive candidates for CNS repair (Temple, 2001). In experimental models, NSC-based therapies have been developed for nervous system disorders, such as stroke, Parkinson's disease, Huntington's disease, and spinal cord injury (Martino and Pluchino, 2006).

Another potential application of NSCs is as gene-delivery vehicles for therapeutic genes. NSCs are ideal in this respect for the treatment of many neurological diseases because of their remarkable migration capacity and their innate tropism for brain pathologies (Lindvall et al, 2004; Muller et al, 2006). NSCs are the preferred vectors used in glioma gene therapy because of their inherent glioma-specific tropism and may overcome the low efficiency of viruses and liposomes.

1.3.3 Neural stem cells: specific glioma tropism property

Both exogenous and endogenous NSCs show unique tropism toward gliomas.

1.3.3.1 Exogenous neural stem cells

Using an implanted brain tumor model in nude mice, Aboody and colleagues first reported the extensive homing ability of NSCs and illustrated that NSCs could deliver therapeutic genes to malignant cells in the brain (Aboody et al, 2000). When injected directly into the intracranial tumor, NSCs not only distributed themselves extensively throughout the main tumor bed, but also

migrated in juxtaposition to individual tumor cells migrating away from the tumor mass and infiltrating into the normal tissue. After intracranial implantation at a distance from the tumor site, such as in normal tissue in the same hemisphere, the contralateral hemisphere, and the cerebral ventricles, NSCs migrated through normal tissue and homed in on the transplanted brain tumor cells. Interestingly, NSCs could target the brain tumor even after intravascular administration. When using NSCs to deliver the therapeutic gene (cytosine deaminase), tumor bulk was reduced and survival improved in mice bearing tumors. In a second report published at the same time, NSCs were used to deliver interleukin-4 in gene therapy of experimental brain tumors (Benedetti et al, 2000). The findings indicated that NSCs engineered to express antitumor genes might be used to track and destroy brain tumors.

1.3.3.2 Endogenous neural stem cells

NSCs share a variety of similarities with brain tumor cells, including the capacity for migration, infiltration into normal brain tissue, and self-renewal, as well as a molecular signature. It has been hypothesized that endogenous NSCs are involved in the development of brain tumors, providing multiple neural lineage cell types (Fomchenko and Holland, 2005). The extensive glioma tropism of endogenous NSCs has been reported in mice (Glass et al, 2005). In elderly mice, endogenous NSCs migrated from the subventricular zone to the grafted tumor, and the NSC accumulation in the tumor site decreased in conjunction with increased tumor size and shorter survival times.

Moreover, coculture of NSCs and glioma cells resulted in the apoptosis of glioma cells.

1.3.4 Mechanism of glioma tropism

The mechanism of NSCs' glioma tropic behavior is not well understood, but it seems that their glioma-specific migration is mediated by multiple cell-surface receptors and secreted proteins. NSCs express a wide variety of receptors. These receptors modulate the migration of NSCs to glioma and enable NSCs to respond to factors released by glioma cells, the tumor stroma (composed of adjacent reactive astrocytes, microglia, oligodendrocytes), the tumor-derived endothelium, and the damaged surrounding normal brain. Several cytokines, chemokines, growth factors, and their receptors have been reported to regulate the migration of NSCs *in vitro* and *in vivo*; these include stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) (Allport et al, 2004; Ehtesham et al, 2004; Imitola et al, 2004), stem cell factor/c-kit (Erlandsson et al, 2004; Sun et al, 2004), HGF/c-Met (Heese et al, 2005), VEGF/VEGFR (vascular endothelial growth factor/receptor, Schanzer et al, 2004; Schmidt et al, 2005), MCP-1/CCR2 (Ji et al, 2004; Widera et al, 2004), and HMGB1/RAGE (Palumbo and Bianchi, 2004; Palumbo et al, 2007). It has been proposed that at least three important physiological processes influence the migratory behavior of transplanted NSCS: inflammation; reactive astrocytosis; and angiogenesis (Muller et al, 2006). In these processes, microglia, astrocyte, and endothelial cells are activated and secrete cytokines,

chemokines, and growth factors to attract NSCs. Extracellular matrix proteins might also contribute to the glioma tropism of NSCs (Ziu et al, 2006). Understanding of the molecular mechanism of NSC migration toward glioma is still quite limited. Additional research is needed to find other molecules with the potential to regulate glioma tropic behavior.

1.3.5 Advantages of neural stem cell vectors in glioma gene therapy

The infiltrative nature of glioma has led to the failure of viral and chemical vectors in clinical trials. Because the distribution of these vectors in brain tumors is limited, only the tumor cells surrounding the injection site are transfected; thus, individual cells migrating from therapeutic areas later give rise to secondary tumors. The unique features of NSCs make them well suited for glioma therapy and may enable them to overcome the limited therapeutic effects of viral and chemical vectors.

NSCs can home in on the main tumor bed and invade single tumor cells over great distances to target the therapeutic genes at the tumor site. Stem cell–based gene therapy may remove the significant obstacle that current glioma therapy faces: the recurrence of secondary tumors due to escaped tumor cells. After genetic modification, NSCs can express transgenes or become vector-producing cells that can deliver therapeutic genes or viruses coding the therapeutic genes.

NSCs might also be able to deliver antitumor transgenes even after systemic administration. The BBB impedes successful delivery of molecules to the CNS neoplasm. However, when NSCs are injected into the cerebral ventricles or even into peripheral circulation, the BBB does not affect the capacity of NSCs to home in on CNS-malignant cells. Tail vein–injected murine NSCs migrated to implanted intracranial gliomas and single infiltrating glioma cells in a manner similar to that of intracranially implanted NSCs (Aboody et al, 2000). Systemic administration of NSCs could eliminate the physiological damage caused by intracranial surgery, and therapeutic effects might be further improved by repeated treatment.

Besides the antitumor effect of therapeutic genes, NSCs themselves can inhibit the tumor growth. *In vitro*, apoptosis of glioma cells has been observed when cocultured with murine NSCs (Glass et al, 2005). In mice, NSCs without a therapeutic gene had a tumor-inhibitory effect and extended the survival of animals with pretransplanted gliomas (Benedetti et al, 2000). The innate tumor-killing capacity of NSCs has also been demonstrated in a rat model (Staflin et al, 2004). Rat neural progenitor cell lines HiB5 and ST14A inhibited tumor growth after coinjection with rat glioma cells into the rat brain.

In addition to their glioma tropic behavior and capacity to cross the BBB and kill tumors, the “stemness” of NSCs makes them more suitable gene-delivery vehicles than other cell types (ie, fibroblast cells). NSCs may integrate

seamlessly into the host brain and repair the CNS damage associated with brain tumors. After implantation, NSCs could generate neurons and glias while stimulating the endogenous repair and neurogenic pathways to promote self-repair in the host (Ourednik et al, 2002; Park et al, 2002). Moreover, self-renewing NSCs can be propagated for long periods in cell culture, fulfilling the requirements of *ex vivo* genetic modification and selection.

1.3.6 Stem cell–based glioma gene therapy

NSCs have a great capacity to home in on brain tumors and show a specific affinity for invading glioma cells. Taking the advantages of inherent tumor tropic, tumor killing and CNS damage repairing properties, researchers have genetically engineered NSCs to selectively deliver various antitumor gene products to disseminating tumors. Gene products, including prodrug-converting enzymes, immunomodulatory cytokines, cytokines with direct antitumoral activity, and proteins with antiangiogenic activity, have been examined in preclinical studies (Muller et al, 2006). Besides, NSCs may be modified into virus-producing cells that can deliver oncolytic viruses directly.

1.3.6.1 Therapeutic genes

1.3.6.1.1 Prodrug-converting enzymes

The prodrug-converting enzyme/prodrug system is also called suicide gene therapy because the enzyme converts the nontoxic prodrugs into an active toxin and causes cell death. NSCs expressing prodrug-converting enzyme in

conjunction with the prodrug reduced tumor growth in animal models bearing glioma and extended the survival of mice. The NSCs' glioma tropism ensures a high concentration of prodrug-converting enzyme in the tumor cell region, not only in the main tumor bulk, but also in glioma cells escaping the tumor bulk. Systemically administered prodrug reaches the brain and is activated by the converting enzyme expressed by the NSCs in juxtaposition to the tumor cells. One of the most attractive advantages of suicide gene therapy is its so-called "bystander effect", meaning that the tumor cells without transgene expression will also be killed by the activated prodrug. The activated prodrug is further transferred from a small number of killed tumor cells to "bystanders," causing tumor-cell death in an even broader region. Therefore, the 'by-stander effect' amplifies the therapeutic effect by broadening the therapeutic region. Suicide gene therapy improves therapeutic efficacy without introducing additional toxicity. By using NSCs to deliver the suicide gene, the concentration of active drug is increased at the tumor site, but the plasma concentration of active drug or prodrug-converting enzyme does not increase compared with treatment using prodrug alone (Aboody et al, 2008). In respect to the potential tumorigenicity of stem cells, the prodrug-converting enzyme serves as a suicide gene and eliminates NSCs after therapy (Li et al, 2005). Prodrug converting enzymes, thymidine kinase, cytosine deaminase, and carboxylesterase are being studied for use in stem cell–based glioma gene therapy.

Herpes simplex virus-thymidine kinase/ganciclovir (HSVtk/GCV) is the most widely used suicide gene therapy and has been best characterized in clinical trials of malignant glioma gene therapy (Pulkkanen and Yla-Herttuala, 2005). In the HSVtk/GCV suicide gene therapy system, the systemic nontoxic GCV passes through the BBB and is converted to active drug by HSVtk in NSCs. The phosphorylated GCV (analog of deoxyguanosine) incorporates itself into the replicating DNA, causing chain termination and cell death. The phosphorylated GCV can pass through gap junctions between adjacent cells and kill the surrounding actively dividing tumor cells. In rat glioma models, HSVtk-transduced primary rat NSCs were intracranially implanted either together with the tumor cells or in the hemisphere contralateral to the tumor site. After the administration of GCV, intracranial tumor growth was markedly inhibited, and survival was significantly prolonged through the bystander effect mediated by HSVtk-transduced NSCs (Li et al, 2007; Li et al, 2005).

The *E. coli* cytosine deaminase (CD)/5-FC system has been tested successfully on murine NSC line C17.2, rat NSC line ST14A, and human NSC line HB1.F3 (Aboody et al, 2000; Aboody et al, 2006b; Barresi et al, 2003; Kim et al, 2006; Shimato et al, 2007). The rabbit carboxylesterase (CE)/ CPT-11 (irinotecan) system has been recently developed and evaluated on HB1.F3 in the treatment of neuroblastoma (Aboody et al, 2006a; Danks et al, 2007).

1.3.6.1.2 Other gene payloads

Interleukins (ie, interleukin-4 [IL-4], interleukin-12 [IL-12], and interleukin-23 [IL-23]) modulate the host immune system to initiate an antitumor response. Genetically modified primary mouse NSCs and rat NSCs expressing IL-4 were tested for efficacy in glioma treatment, resulting in prolonged survival of the treated rats compared with untreated controls (Benedetti et al, 2000). In another study, implantation of NSCs expressing IL-12 in rats bearing GL261 tumors extended survival and produced long-term antitumor immunity (Ehtesham et al, 2002b). Another interleukin, IL-23, has been delivered by bone marrow–derived NSC-like cells (BM-NSCs) and showed an antitumor effect in a mouse model with glioma (Yuan et al, 2006).

Tumor necrosis factor–related apoptosis inducing ligand (TRAIL), a proapoptotic protein in the tumor necrosis family, induces apoptosis selectively in tumor cells in animal models (Walczak et al, 1999). Inoculation of TRAIL-secreting NSCs caused tumor apoptosis and reduction in nude mice bearing human U343 glioma xenografts (Ehtesham et al, 2002a).

Interferon-β (IFN-β) can inhibit tumor growth by indirect immunomodulatory and antiangiogenic properties or by direct antiproliferative effects on malignant cells (Studeny et al, 2002). Human NSC line HB1.F3 was exploited to target delivery of IFN-β to disseminated neuroblastoma (Dickson et al, 2007). Intravascularly administrated IFN-β-HB1.F3 cells significantly delayed

tumor growth and might circumvent limitations associated with the systemic toxicity of IFN-β. Bone marrow–derived MSCs could also serve as vehicles for IFN-β delivery into intracranial glioma and melanoma lung metastasis, resulting in tumor inhibition and prolonged survival that could not be achieved by systemic administration of IFN-β (Nakamizo et al, 2005; Studeny et al, 2002).

PEX, a natural fragment of human metalloproteinase-2, inhibits glioma angiogenesis, cell proliferation, and migration (Bello et al, 2001; Brooks et al, 1998). In nude mice with U87 gliomas, PEX-producing human NSC HB1.F3 reduced tumor volume by 90% after intratumoral implantation (Kim et al, 2005). The tumor reduction was associated with a significant decrease in angiogenesis and proliferation.

Oncolytic viruses also can be targeted to brain tumors by using the NSC itself to produce and deliver the viral particles to widespread tumor cells. Murine NSCs were used to produce and deliver replication-conditional HSV-1 (Herrlinger et al, 2000). Tumor growth in mice bearing glioma was inhibited by NSC-released HSV-1, thus overcoming the typical hurdle of low transduction efficiency that impedes virus-based glioma gene therapy.

1.3.6.2 Cell sources

To date, one of the major limitations of stem cell–based glioma gene therapy is the use of fetal-derived NSCs. Human NSCs used in experimental models are mainly isolated from the fetal human brain by FACS (Rossi and Cattaneo, 2002). The process is quite tedious and NSCs are difficult to expand after isolation. Moreover, the fetal source of NSCs raises serious ethical and legal concerns. To overcome these hurdles, alternative large-scale sources of NSCs must be identified (Martino and Pluchino, 2006).

Primary adult NSCs may be a viable choice in terms of the similarity between primary NSCs and endogenous NSCs, as well as the encouraging results seen in primary rodent NSC-mediated glioma gene therapy (Ehtesham et al, 2002a; Ehtesham et al, 2002b; Li et al, 2007; Li et al, 2005; Shah et al, 2005), but unlike the isolation of HSCs from bone marrow, the procedure to derive primary NSCs from the adult brain is extremely invasive and offers a low yield. In cell culture, human adult NSCs express low levels of telomerase and stop proliferation after serial passaging (Ostenfeld et al, 2000), so optimized protocols to expand adult NSCs *in vitro* will be required. In addition, because the behavior of NSCs derived from diverse sources and maintained under different culture conditions may vary *in vivo*, researchers should establish standards for the isolation, expansion, and characterization of adult NSCs.

Immortalized NSC lines (eg, v-*myc* immortalized mouse fetal NSC line C17.2 and human fetal NSC line HB1.F3) have shown outstanding migratory capacity and antitumor effect in a variety of experimental brain tumor models (Aboody et al, 2000; Aboody et al, 2008; Aboody et al, 2006b; Danks et al, 2007; Dickson et al, 2007; Herrlinger et al, 2000; Kim et al, 2005; Kim et al, 2006; Shimato et al, 2007). Compared with primary NSCs, immortalized NSC lines are well characterized and can be propagated indefinitely with defined properties on a large scale. Hence, by comprehensive analysis on cell lines, the quality of cells used in implantation can be easily controlled. But the utility of oncogenes during immortalization and the potential tumorigenicity of cell line *in vivo* raise safety concerns. Furthermore, the transplantation of allogeneic NSC lines may cause immune rejection, although NSCs have been reported to have low immunogenic potential in their undifferentiated state (Hori et al, 2007).

Human ESC-derived NSCs might provide an unlimited cell source for therapeutic applications. They also offer several advantages over other types of stem cells. Self-renewing ESCs are inherently immortal and their proliferation capacity is preserved during long-term cell culture. So far, 21 independent human ESC lines, characterized by the National Institutes of Health (NIH) using universally accepted criteria, are commercially available worldwide, suggesting that all labs can start from the same cell populations. The great plasticity of ESCs allows the derivation and isolation of glioma

tropic NSCs, which can serve as targeting gene-delivery vehicles in the treatment of patients with malignant glioma. Several strategies have been developed to differentiate NSCs from human ESCs. ESC-derived NSCs have shown extensive migratory ability, differentiating into neurons, astrocytes, and oligodendrocytes in normal and lesioned rat brains (Tabar et al, 2005). Additional work should compare the migratory potential of human ESC-derived NSCs with other NSCs to gliomas *in vitro* and *in vivo* and further explore the possibility of ESC-derived NSCs as antitumor gene-delivery vectors for glioma therapy. However, the tumorigenicity of undifferentiated ESCs is a serious concern, and the protocol to avoid teratoma formation *in vivo* after transplantation of ESC-derived cells is still not available (Martino and Pluchino, 2006). Immunogenicity might be another problem with ESC-derived NSCs, as it is in immortalized cell lines. Since the first derivation of human ES cell line in 1998, debate on the embryo destruction involved in the development of human ES cell lines has never stopped. To circumvent this ethical issue, a number of alternative sources of ESCs are being investigated (eg, single blastomeres, growth-arrested embryos, and somatic cells through somatic cell nuclear transfer and cellular reprogramming) (Klimanskaya et al, 2008). Recent scientific breakthroughs in deriving induced pluripotent stem (iPS) cells by cellular reprogramming of adult human fibroblasts opens a new window on stem cell research.

Induced pluripotent stem (iPS) cells may succeed ESCs in the near future in clinical therapeutic applications. Reprogramming to a pluripotent state was first achieved in mouse tail fibroblasts in 2006 by overexpressing four transcription factors, Oct4, Sox2, Myc, and Klf4 (Takahashi and Yamanaka, 2006). Until the end of 2007, two groups had reported successful reprogramming of human somatic cells to iPS cell lines with defined transcription factors, either Oct4, Sox2, Myc, and Klf4 (Takahashi et al, 2007), or another set, Oct4, Sox2, Nanog, and Lin28 (Yu et al, 2007). This novel cellular reprogramming technique allowed the derivation of patient-specific pluripotent stem cells from their own somatic cells, thus avoiding ethical issues. Current studies support that, based on the characterization of morphology, chromosome profile, and gene expression profile of human iPS cells, there is no major difference between human iPS cells and human ESCs (Cyranoski, 2008; Lowry et al, 2008). NSCs derived from iPS cells might provide an autologous cell source for glioma gene therapy, possibly removing the immune rejection induced by other type of stem cells. More effort should be focused on testing the differentiation protocols of human ESCs in iPS cells and comparing the function and behavior of the derivatives of ESCs and iPS cells *in vivo*.

MSCs might be another vector candidate for gene delivery in glioma gene therapy. In glioma models, MSCs exhibited a migratory capacity similar to that of NSCs toward tumor cells and inhibited tumor growth when expressing

antitumor genes (Miletic et al, 2007; Nakamizo et al, 2005; Nakamura et al, 2004; Studeny et al, 2002).

1.4 Purpose

Via two main objectives, this study aims to improve the treatment of patients with malignant glioma and overcome hurdles faced by glioma gene therapy in clinical trials.

1) To discover new regulators that enhance cell migration toward gliomas once overexpressed. Manipulation of the expression of these molecules could then facilitate the use of NSCs as gene-therapy vectors to reach scattered glioma cells.
2) To develop new sources of NSCs and thus overcome the source limitations and ethical issues of NSCs isolated from fetal brains. To achieve this, we derived glioma tropic stem cells from human ESC lines and human neural precursor cell (NPC) lines.

In Chapter 2, we used Boyden chambers to select the cells that were primed by gene transfer of a tumor cDNA expression library. A novel gene encoding, transmembrane protein 18 (TMEM18), emerged from the screen and was selected for extensive characterization. The regulatory effects of TMEM18 on the glioma tropism of NSCs were studied after overexpressing and siRNA silencing. The mechanism of TMEM18-mediated glioma-specific migration of NSCs also was explored.

In Chapter 3, the glioma tropic stem cells were derived from human neural precursor cell (NPC) line NTera2/D1 (NT2) by controlled differentiation. The neural differentiation of NT2 was induced by 2-week retinoic acid (RA) treatment, and glioma tropic precursor cells were isolated using the Boyden chamber migration screening. The migration capacity of the glioma tropic precursor cells (NT2.RA2 migrating cells) toward glioma cells was studied *in vitro* and *in vivo*. The antitumor effect of NT2.RA2 migrating cells expressing therapeutic gene HSVtk was further investigated in nude mice.

In Chapter 4, ESC lines HES1 and HES3 were differentiated to NSCs (named NSC1 and NSC3, respectively) in an adherent monolayer culture. NSC1 and NSC3 cells could be expanded easily in defined medium while maintaining their neural multipotency. In a Boyden chamber migration assay, we compared the migration of NSC1 and NSC3 cells with that of mouse NSC line C17.2, which had already been well studied. The *in vitro* therapeutic effects of NSC1 cells as gene-delivery vectors for glioma gene therapy were also examined.

This thesis describes, for the first time, stem cells derived from human ESCs and human neural precursor NT2 cells as gene-delivery vehicles in glioma gene therapy. By using these novel stem cell vectors, efficient and targeting glioma gene therapy may be achieved. Furthermore, this study could indicate

an unlimited new clinically viable cell source, considering the long-term proliferation of the stem cells. When combined with the new somatic cell–reprogramming technique, we may able to develop patient-specific stem cell–based gene therapy. The research on TMEM18 broadens the scope of understanding of glioma tropism, and overexpression of this protein could be favorably used in stem cell–based glioma gene therapy. In the future, we will use the TMEM18 genetic-engineering strategy together with ESC-derived stem cells to further improve efficiency. Hopefully, our efforts will lead to improvements in glioma therapy, prolonging the survival of patients with malignant glioblastoma.

CHAPTER 2

TRANSMEMBRANE PROTEIN 18 ENHANCES THE TROPISM OF NEURAL STEM CELLS FOR GLIOMA CELLS

2.1 Introduction

Glioma cells misregulate the expression of growth factors, proteases, and extracellular matrix and cell surface proteins to gain their devastating invasion capacity (reviewed by Tysnes and Mahesparan, 2001; Mueller *et al*, 2003). Localized treatments are thus inefficient and comprehensive treatments are too damaging to the delicate brain. A solution is to find a treatment that can specifically locate the tumor cells. Neural stem and precursor cells have an intrinsic tropism for sites of brain injuries including gliomas and, as demonstrated first by Benedetti *et al*. (2000) and Aboody *et al*. (2000), engrafted primary and immortalized neural stem cells can be used in gene therapy of gliomas in animal models. These engrafted stem cells have been shown to spread through the existing migratory pathways in healthy brain as well as non-typical routes when gliomas are present (Flax *et al*, 1998; Aboody *et al*, 2000). Besides primary and immortalized neural stem/precursor cells, embryonic stem cell–derived neural progenitor cells seem to have the same aptitude for glioma cell tracking (Arnhold *et al*, 2003). Moreover, neural stem cells are able to locate not only gliomas but also tumors of a non-neural origin, suggesting that there exist common regulators of cell trafficking probably composed of secreted factors from a tumor site and receptors present on neural stem cells (Brown *et al*, 2003; Allport *et al,* 2004).

Candidate signals to attract neural stem cells to the sites of brain injuries and tumors have been studied. Among them are cytokines released

from brain's immunoreactive migroglial cells during inflammation (Aarum *et al*, 2003), which also provide cues for neural stem cell migration in brain development (reviewed by Tran and Miller, 2003). Stromal Cell Derived Factor 1 (SDF-1) chemokine can attract neural stem cells too. When its receptor CX Chemokine Receptor 4 (CXCR4) is blocked, SDF-1 hinders the neural stem cell migration to the site of injury (Aarum *et al*, 2003; Imitola *et al*, 2004; Ehtesham *et al*, 2006). Also, chemokine Monocyte Chemoattractant Protein-1, which expression can be induced by Tumor Necrosis Factor-alpha, can activate migration of neural stem cells (Widera *et al*, 2004). Cytokine Stem Cell Factor, expressed by glioma cell lines and overexpressed in neurons at the sites of brain injury, is another possible contributing attractant for neural stem cells (Erlandsson *et al*, 2004; Sun *et al*, 2004; Serfozo *et al*, 2006). Similar to cytokines and chemokines, growth factor-mediated signaling, for example Vascular Endothelial Growth Factor and Epidermal Growth Factor Receptor, has been shown to regulate neural stem and progenitor cell migration (Boockvar *et al*, 2003; Schmidt *et al*, 2005). Glioma invasion depends largely on the cell ability to modify the extracellular matrix and interestingly the extracellular matrix secreted from glioma cell lines is able to promote neural stem cell motility (Ziu *et al*, 2006).

The picture emerging from the above studies seems to support a model of the complex interaction of several factors in regulating neural stem cell migration toward tumors. We hypothesized that other regulators are likely

to exist and their genes can be identified through expression cloning on the basis of gene function in influencing the tropism of neural stem cells toward glioma cells. We were particularly interested in the molecules that once over-expressed in neural stem and precursor cells are able to enhance cell migration toward gliomas, as the manipulation of the expression of these molecules could then facilitate the use of neural stem/precursor cells as gene therapy vectors to reach scattered glioma cells. We used Boyden chambers in the current study to select the cells that were primed by gene transfer of a tumor cDNA expression library. A novel gene encoding Transmembrane Protein 18 (TMEM18) emerged from the screen and was selected for extensive characterizations. We demonstrate that TMEM18 is an endogenous regulator of general motility of neural stem cells and that once over-expressed it provides the cells an improved preference for glioma cells.

2.2 Materials and methods

2.2.1 Cell culture

NT2, U87MG, H4, and NIH3T3 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, Virginia) and HEK 293FT cells were purchased from Invitrogen (Carlsbad, CA). All the cell lines were maintained in DMEM supplemented with 10% fetal calf serum (Gibco Life Technologies, Gaithersburg, MD), penicillin-streptomycin (Gibco), normoxin (Invivogen, San Diego, CA), and Non-essential amino acids (Gibco). C17.2 cells were kindly provided by Prof E. Arenas, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden and were maintained in DMEM supplemented with 10% fetal calf serum (Gibco), 5% horse serum (Gibco), penicillin-streptomycin (Gibco), normoxin (Invivogen), and Non-essential amino acids (Gibco). The National Institutes of Health (NIH) Human Embryonic Stem Cell Registry listed hES cell line, HES-1, and its feeder cell K_4 mouse embryonic fibroblasts (mEFs) were obtained from ES Cell International (ESI), Singapore. The hES cells were amplified and maintained according to the protocol provided by ESI. Embryoid boides and neural spheres from hES cells were generated as previously described (Zeng *et al*, 2007).

Primary murine NSCs and NPCs were isolated from the embryonic forebrain of C57BL/6 mice. The treatment of animals was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of our institution.

Pregnant C57BL/6 mice at the specified gestational age of 14 d (E14) were killed via cervical dislocation and the uteri were aseptically removed. Fetuses were removed from the amniotic sac and transferred to a Petri dish containing ice-cold HBSS. Cortices were rapidly excised from the fetuses and mechanically dissociated by pipetting into a single-cell suspension. Cells were plated at a density of 2 x 10^5/mL into 10-cm culture dishes (Nunc) in DMEM/nutrient mixture F-12 (1:1) mixture medium (Invitrogen) containing B27 supplement (Invitrogen), 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich), 20ng/mL EGF (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). Floating neurospheres with diameter range between 150 and 250 Am were passaged every 6 to 7 d. The multipotency of the cells was confirmed by immunocytochemical analysis after differentiation into three fundamental lineages in central nervous system (neurons, astrocytes, and oligodendrocytes).

2.2.2 cDNA expression library screening

Human Daudi cell cDNA library containing retrovirus supernatants were purchased from Stratagene (La Jolla, CA) and used as recommended by the supplier. One million of NT2 cells were infected with the cDNA library retrovirus supernatants to yield 20% infection efficiency in order to ensure a proper presentation of all the cDNAs in the library. Cells were allowed to recover for 4 days, after which they were selected in transwell migration assays using Boyden chambers as described below. Migrating cells were

collected and let recover for 5 days before the next migration assay. After three rounds of the migration assays, both non-migrating and migrating cells were collected.

For analysis of virus-imported cDNAs, chromosomal DNA was purified from non-migrating or migrating cells using DNeasy^{TM} kit (Qiagen, Hilden, Germany) as recommended by the manufacture. Retrovirus imported sequences were recovered according to a PCR protocol suggested in ViraPort manual (Stratagene). Same amount of chromosomal DNA was used in PCR for non-migrating and migrating cells. The success of the PCR was verified by running aliquots of the reactions on an agarose gel. PCR products were subsequently cloned into pDrive using TA-cloning kit (Qiagen). DH5α *E. coli* cells were transformed with the cloning products and plated. After overnight incubation, bacterial clones were picked and plasmid DNA was isolated, and then subsequently used in PCR using the same conditions as previously described to isolate individual sequences for sequencing.

2.2.3 Overexpression and gene silencing

TMEM18 was cloned from Human Daudi cell cDNA library infected cells by PCR using primers 5' caccatgccgtccg ccttctctg and 5' aaagtcttctttc cttctccttttc into pLenti6/V5-TOPO vector (Invitrogen), followed by sequencing to ensure that the cloned sequence was correct. TMEM18A virus contained one amino acid mutation from alanine to threonine at position 103, which did not seem to

have any effect in later experiments. Empty and TMEM18 Lentiviruses were produced using ViraPower™ Lentiviral Directional TOPO® Expression Kit respectively (Invitrogen). Virus transduction in NT2 and C17.2 cells to express TMEM18, cell selection for stable expression and cell maintenance were carried out as recommended by the manufacturer (Invitrogen). The titer of lentivirus infection was controlled to have one virus per cell.

For TMEM18 overexpression in primary murine NSCs/NPCs, neural spheres were dissociated on the day of transfection, and plasmid DNA pLenti6/V5-TMEM18 was transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. pLenti/V5-eGFP plasmid was used as a vector control. Thirty hours after the transfection, cells were collected for the migration assay and reverse transcription-PCR (RT-PCR) study described below.

For siRNA-mediated *TMEM18* gene silencing, two sequences, 5'-tcatcttagtctactgtgctgaata and 5'-tgctcacgcagacggactggactga, were cloned into double promoter siRNA expression vector pFIV-H1/U6-PURO (System Biosciences, Mountain View, CA) as recommended by the manufacturer's protocol. A siRNA sequence against *luciferase* provided in pFIV-vector cloning kit (System Biosciences) was used as a control. Cells were plated to reach 90% confluence on the day of transfection of the siRNA expression plasmids, and plasmid DNA was transfected with Lipofectamine 2000

(Invitrogen) according to the manufacturer's protocol. Puromycin resistant cells were selected for 4 days, after which cells were used for migration assay and for RT-PCR study.

2.2.4 RT-PCR

Cytoplasmic RNA was collected with RNeasy Kit^{TM} (Qiagen) as recommended by the manufacturer. The concentration and purity was verified before equal amounts of RNAs from all the samples were used to produce cDNAs by reverse transcription using oligo-T-priming of Superscript III First-Strand Synthesis System (Invitrogen). PCR amplification for the produced cDNAs was carried out using HotStart Taq system (Qiagen) as suggested by the HotStart Taq manual. Real-time PCR was done using Power Sybr Green PCR master mix and protocol (Applied Biosystems, Foster City, CA), with primers for TMEM18 5'-atg ccg tcc gcc ttc tct g and 5'-gtc ttc ttt cct tct cct ttt c, and primers for beta-actin 5'-tcatgtttgagaccttcaa and 5'-gtctttgcggatgtccacg. Opticon 2 real-time PCR machine (Applied Biosystems) was used to run the PCR reactions. The program for TMEM18 PCR was 10 minutes at 95°C, followed 45 cycles of 15 seconds at 95°C followed by 1minute at 68°C; and the program for beta-actin was 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 55°C, and 20 seconds at 68°C. Real-time PCR results were presented as a ratio of TMEM18 mRNA to beta-actin mRNA.

2.2.5 Immunostaining and Western blot analysis

Antibody against TMEM18 was produced in rabbits against peptide 122-135: C-DLKNAQERRKEKKR (Biogenes GmbH, Berlin, Germany). This peptide is unique to TMEM18 based upon BLAST search. The anti-TMEM18 serum was tested using Western blotting and detected one band with molecular mass of 18 kDa, which was blocked when the serum was incubated in the presence of the immunizing peptide. The serum was used in 1:200 for immunostaining and Western blot analysis. Antibody against alpha-tubulin (ab7291) was purchased from Zymed (South San Francisco, CA) and used in 1:100 in immunofluorescence. Secondary fluorescence antibodies were purchased from Jackson immunoresearch (West Grove, PA). 4',6-Diamidino-2-phenylindole (Invitrogen-Molecular Probes, Eugene, OR), DAPI, was used in concentration of 2 nM.

2.2.6 *In vitro* cell migration assay

In vitro migration of neural progenitor cells toward glioma cells was examined using Boyden chamber assays. A migration kit from BD Falcon (Franklin Lakes, NJ) with 24-well cell culture plates was utilized. Each well of the plates was separated into two chambers by an insert membrane of 8 μm pores. One day before assays 50,000 glioma cells were seeded into each lower chamber. The next day cell culture medium in the lower chamber was removed and replaced with 500 μl of non-supplemented DMEM. Neural stem/precursor cells (50,000 in 500 μl of non-supplemented DMEM) were then seeded into

the upper chamber. For the assay using a neutralization antibody to block cell migration, 40 μg/ml of anti-chemokine receptor 4 (CXCR4) monoclonal antibodies (R&D Systems, Minneapolis, MN) was incubated with neural stem and precursor cells for 30 minutes at room temperature prior to the cell seeding. After 12 or 24 hours of incubation at 37°C, migrating cells on the bottom of the insert membrane and non-migrating cells on the upper side of the membrane were dissociated by trypsination. These cells were subsequently lysed and stained using a CyQUANT cell proliferation assay kit (Molecular probes). Flurorescence was measured with a fluorescence plate reader (GENios pro, Tecan, Dorset, United Kingdom). Values from 6 to 12 wells were expressed as the mean ± standard deviation (SD) in percentage control. In most of *in vitro* migration assays, the migration of cells transduced with a vector control in response to serum-free DMEM was used as the basal migration rate. In those experiments without the use of a vector control, cell migration in response to serum-free DMEM was used as the basal migration rate. Statistical analyses were done using Student's t-test.

2.2.7 *In vivo* cell migration assay

Rat C6 glioma cells (1 million cells in 5 μl PBS) were injected into the right striatum of the rat brain (AP+1.0 mm, ML +2.5 mm, and DV -5.0 mm from bregma and dura) using a 10 μl Hamilton syringe connected with a 30-gauge needle at a speed of 0.5 μl/min. Three days later, 1.25 million of green fluorescent DiO dye (Invitrogen)-labeled TMEM18-overexpressing C17.2 cells

were mixed with the equal number of red fluorescent DiI dye (Invitrogen)-labeled vector control C17.2 cells and injected into the contralateral side of the rat brain. The brain samples were collected 3 weeks later for sectioning and examination. To quantify the number of migrating cells in the migration front, red and green fluorescent cells were counted in 10 sections, with dots of yellow color being considered as co-migration of green and red cells. In the handling and care of animals, *the Guidelines on the Care and Use of Animals for Scientific Purposes* issued by National Advisory Committee for Laboratory Animal Research, Singapore was followed. The experimental protocols of the current study were approved by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore and Biological Resource Center, the Agency for Science, Technology and Research (A*STAR), Singapore.

2.2.8 Nuclear localization assay

GFP fusion protein expression plasmids were created as instructed in NT-GFP Fusion TOPO® Expression Kit manual (Invitrogen). The TMEM18 N-terminus with 15 amino acid residues was cloned by PCR using primers 5'-tcagtcttctttccttctcc and 5'-aagaatgcacaagagagaag. TAT coding sequence was formed by annealing oligos 5'-cagcgcaaaaaacgccgccagcgccgctaga and 5'-ctagcggcgctggcggcgtttttgcgctga. Plasmid constructs generated were sequenced to confirm GFP fusion and transfected into U87MG cells by Lipofectamine 2000 (Invitrogen).

2.3 Results

2.3.1 TMEM18 is a novel modulator identified by cDNA expression library screening for the genes that promote glioma-directed stem cell migration

To identify genes that promote the migration of stem cells toward glioma cells, we preformed cDNA expression library screening. A cDNA library derived from the Daudi Burkitt lymphoma cell line was used for expression cloning, in view of the capacity of the cells to invade locally and to metastatize via mechanisms similar to those developed by solid tumors (Makrynikola *et al*, 1994; Vacca *et al*, 1998). Retrovirus vectors were used to transduce the cDNA library into human neural precursor cell line NT2. The transduced cells were evaluated subsequently for their glioma-directed migration ability in a transwell cell migration assay using Boyden chambers. In the assay, non-migrating cells stayed on the top of the membrane, whereas cells that were primed to migrate went through 8 micrometer pores into the opposite site of the transwell insert membrane. Migrated cells were isolated and passed through two more rounds of the migration assay selection, after which virus-imported cDNAs in migrating cells were cloned by PCR and identified by sequencing. The protocol is summarized in Figure 2.1. Non-migrating cells were used as controls for the cDNA analysis.

We sequenced 70 virus imported cDNA clones from migrating cells and 46 clones from non-migrating cells and identified a number of cDNAs that

express proteins capable of enhancing the tropism of NT2 cells for glioma cells. Among the clones collected from the migrating cells for sequence analysis, two were found to encode for TMEM18, while no clone from the non-migrating cells had virus imported *TMEM18* gene sequence. TMEM18 was thus a promising candidate for further analysis for its role in regulating neural stem cell migration.

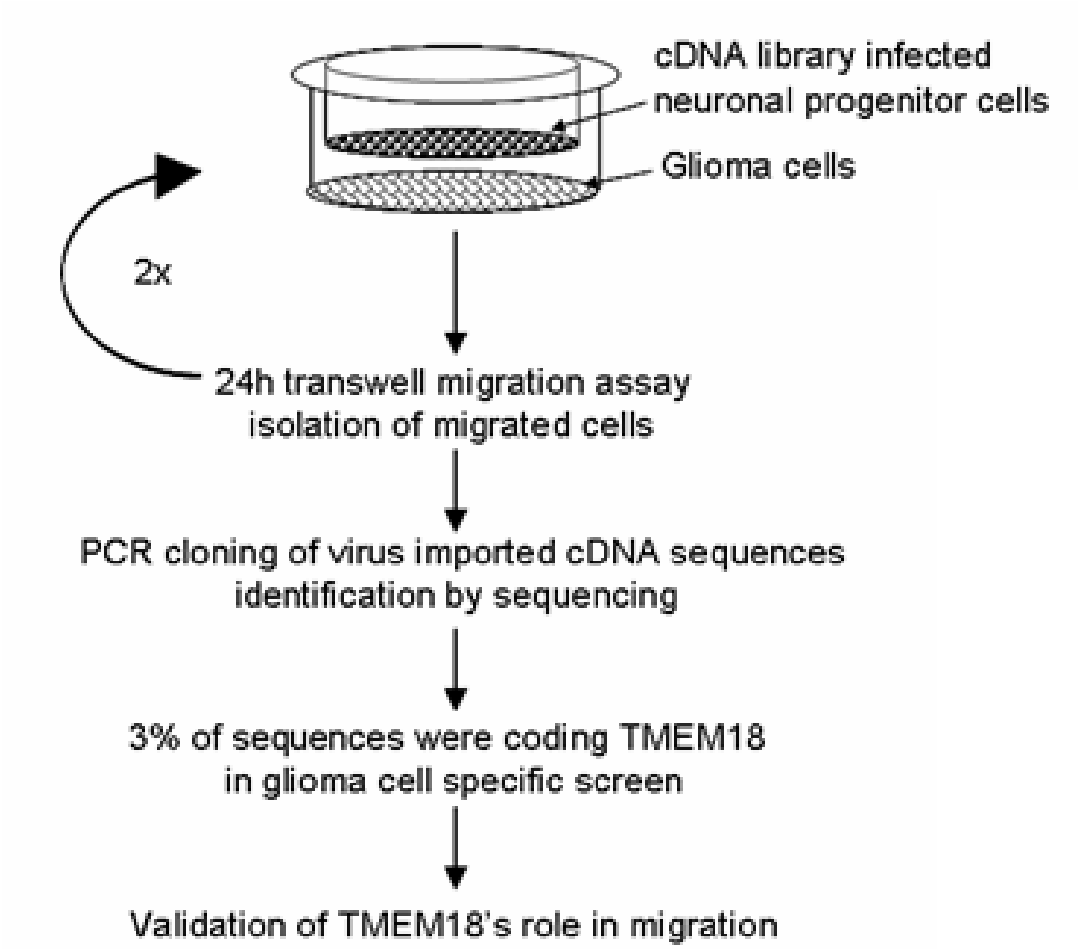


Figure 2.1. Flowchart of cDNA expression library screening to discover novel proteins able to promote neural precursor cell migration toward glioma cells. Human neural precursor cell line, NT2, was infected with retrovirus carrying a cDNA library. Infected cells were plated on transwell migration insert on top of glioma cell culture in a Boyden chamber. The neural precursor cells able to migrate through insert's pores to the opposite site of the insert's membrane were collected and passed two more times through the same migration assay. The virus imported cDNAs in migrated cells were cloned by PCR and sequenced.

2.3.2 TMEM18 is a potential transmembrane protein with a C-terminal nuclear localization signal

The *TMEM18* gene encodes a novel protein with no published reports describing its function so far. When we search for functional motifs in TMEM18 amino acid sequence using several web-based programs (ca.expacy.org/tools/), no strong association with any previously characterized domains was found, though weak potential sites for phosphorylation and N-myristoylation did appear. TMEM18 is predicated to contain four transmembrane spanning alpha-helixes (Figure 2.2) by TMpred, a program designed for identification of transmembrane proteins (Hofmann and Stoffel, 1993), although the first membrane spanning part is less probable than the other three based on some other prediction programs. Using PredictNLS-program (Cokol *et al*, 2000), we noted a putative nuclear localization signal (NLS) sequence (RKEKKRRRK) at the strongly hydrophilic C-terminus of TMEM18 (Fig. 1C underlined sequence). ClustalW-program (ch.EMBnet.org) was used to align TMEM18 protein sequences from human (NCBI accession no. NP_690047.2), mouse (NP_742046.1), rat (NP_001007749.1), dog (XP_848731.1), and chicken (XP_419929.1). The strong conservation across species (Figure 2.3) implies that TMEM18 has a key function(s). Moreover, using National Center of Biotechnology Information (NCBI) search program for homologous protein sequences (Homologene), homologous sequences from fruit fly (*D. melanogaster*) to rice (*O.sativa*) were also found (data not shown).

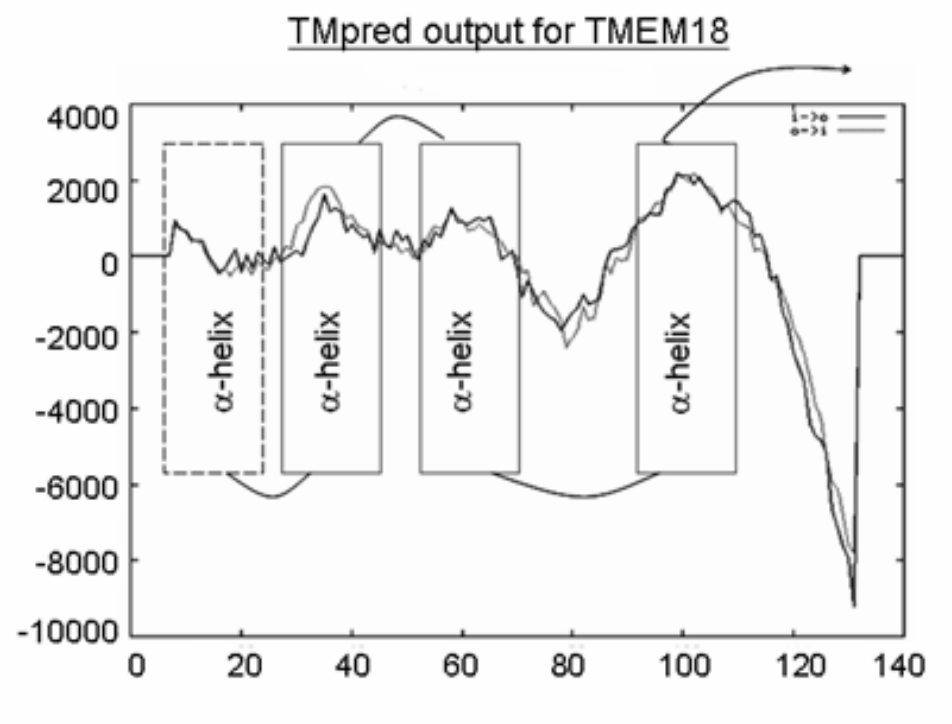


Figure 2.2. TMpred-program predicts TMEM18 to have four membrane spanning alpha-helixes (Hofman and Stoffel, 1993). Schematic interpretation of the TMEM18 protein structure is overlaid with TMpred-graph to illustrate the results from the program.

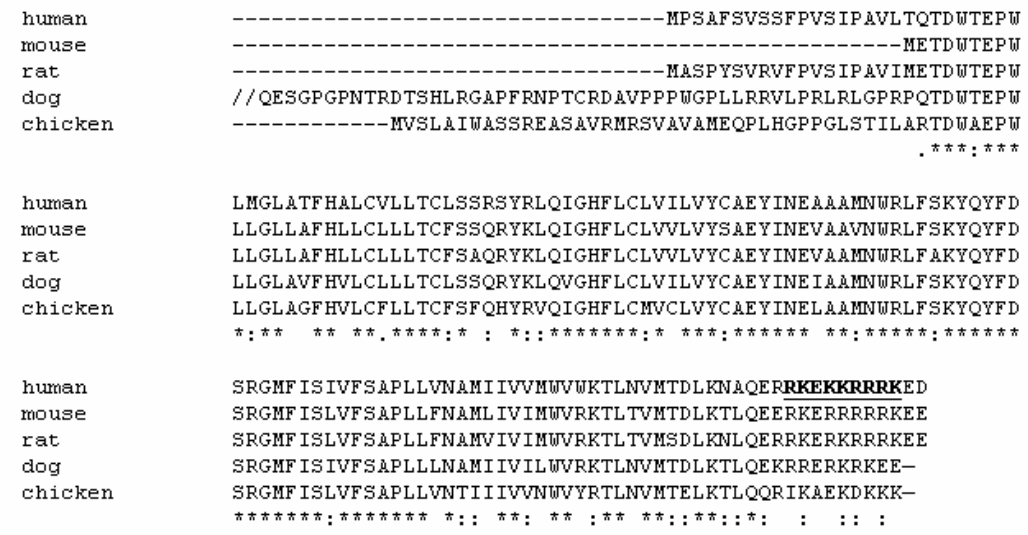


Figure 2.3. Sequence alignment of TMEM18 proteins from human, mouse, rat, dog, and chicken. A star (*) indicates perfect amino acid conservation and a colon (:) one amino acid difference in the sequences. Only the end of C-terminus is shown for the dog sequence. Bold underlined sequence represents possible nuclear localization signal peptides predicted by PredictNLS-program.

2.3.3 Overexpression of TMEM18 enhances the *in vitro* glioma-specific migration ability of neural stem/precursor cell lines

TMEM18 was identified as a promoting factor for neural stem cell migration toward glioma cells in our cDNA expression library screening. To verify the observation, we investigated whether the overexpression of the *TMEM18* cDNA in NT2 human neural precursor cells and C17.2 murine neural stem cells would affect the migration. We used lentiviral vectors to create stable cells lines overexpressing TMEM18 in NT2 and C17.2 cells. Two populations of stable cell lines that express different levels of TMEM18 were selected for each type of neural precursor cells. TMEM18 overexpression was confirmed using RT-PCR (Figure 2.4 A NT2 cells and 2.5 A for C17.2 cells).

To assess the effects of TMEM18 overexpression on cell migration, we used Boyden chamber assay to examine the movement of these cells lines toward human U87MG glioma cells, the same tumor cell line that was used in the cDNA expression library screening earlier. TMEM18-overexpressing NT2 and C17.2 cells displayed significantly higher migration capacities when compared with their parental cells and empty vector controls (Figure 2.4 B & 2.5 B). These cells also responded to other glioma cell lines, H4 and C6, by displaying significant migration advantage over control cells. Interestingly, the TMEM18-overexpressing NT2 cells did not change their migration capacities when non-tumor cell lines, mouse fibroblast cell line NIH3T3 and human kidney cell line HEK 293FT, were seeded in the bottom chamber in the

assays. Moreover, the amount of cells migrating to plain DMEM cell culture medium remained similar between the TMEM18-overexpressing cells and the controls. Hence, the preference of TMEM18-overexpressing cells for glioma cells implies a role for the protein in response to glioma secreted factors.

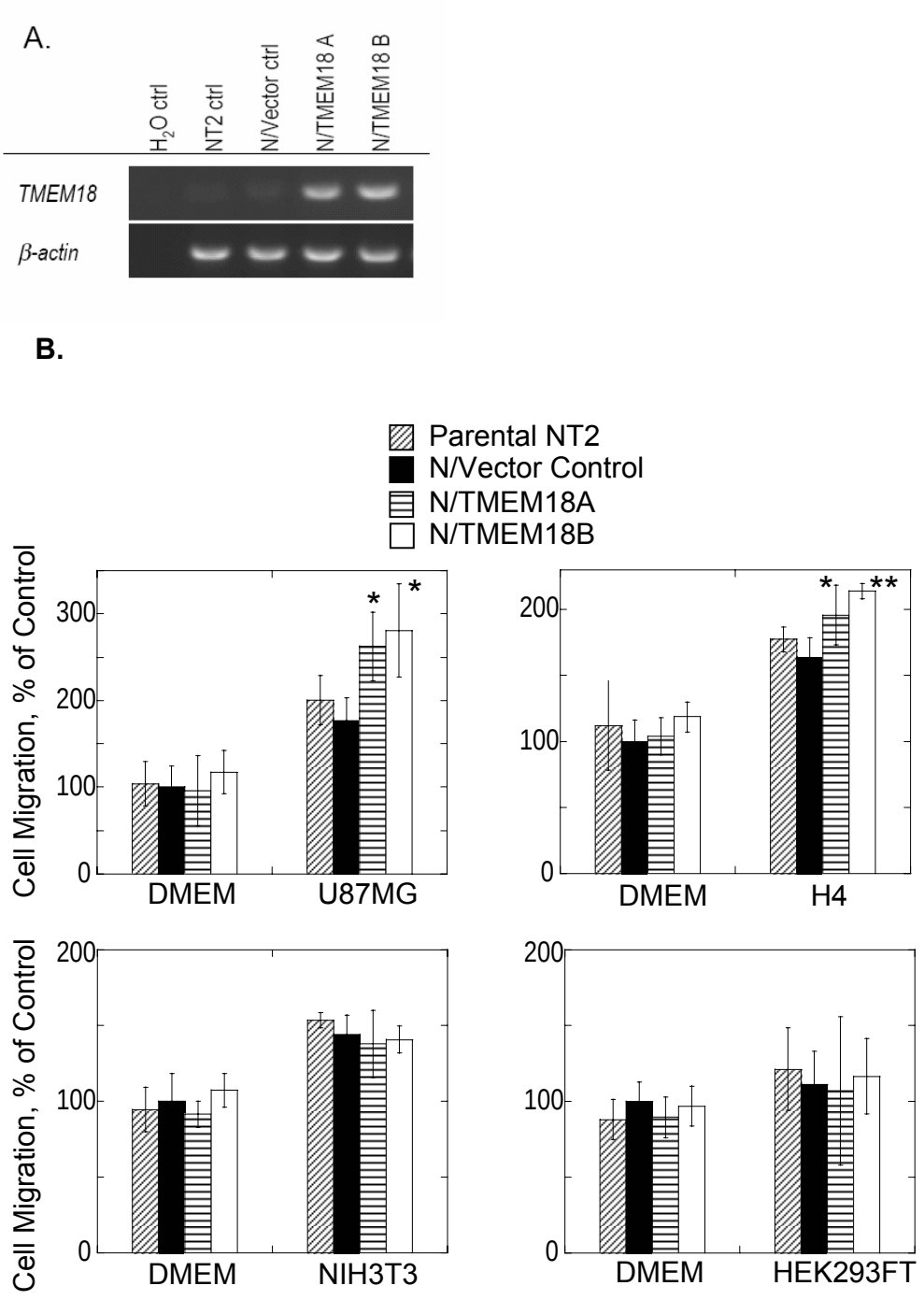
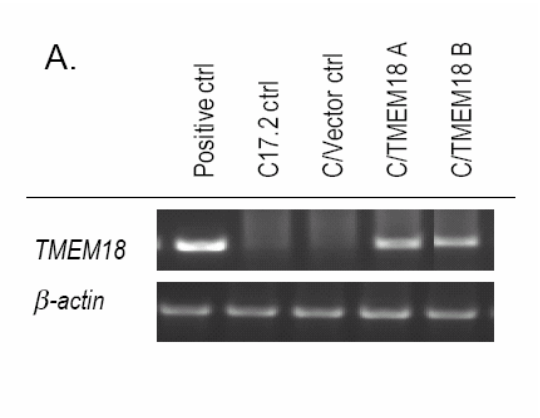


Figure 2.4. TMEM18 overexpression increases the migration activity of human neural precursor NT2 cells in Boyden chamber assays. Lentivirus-mediated TMEM18 overexpression was examined by RT-PCR (A). B. *In vitro* migration of TMEM18 overexpression NT2. Columns, percentage of DMEM control; bars, SD. Statistical analysis to DMEM control is calculated using Student's *t* test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. (Contributed by Dr. Jurvansuu Jaana)



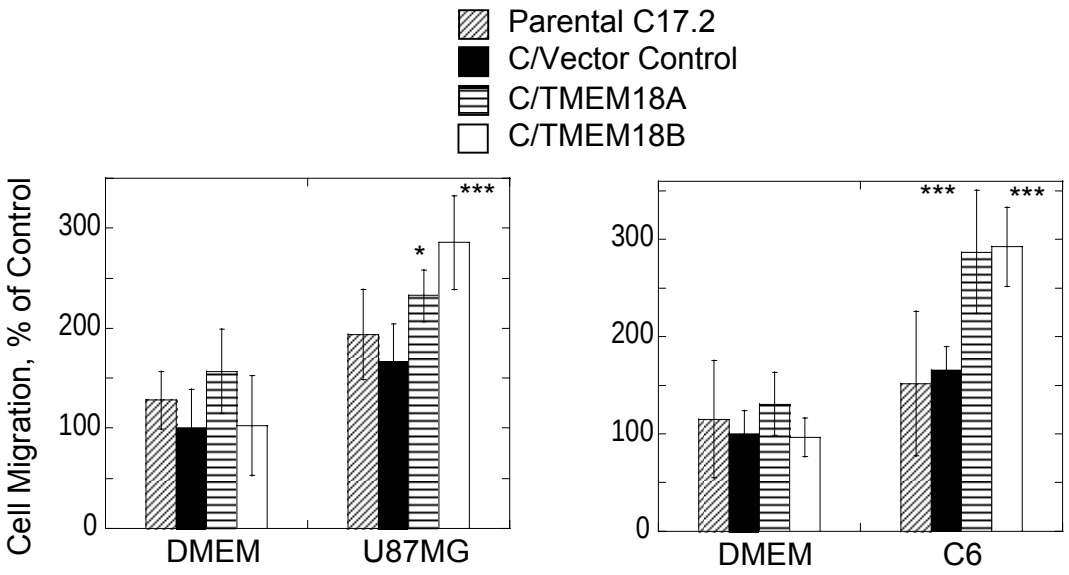


Figure 2.5. TMEM18 overexpression increases the migration activity of mouse neural stem cells C17.2 in Boyden chamber assays. Lentivirus-mediated TMEM18 overexpression was examined by RT-PCR (A). B. *In vitro* migration of TMEM18 overexpression C17.2. Columns, percentage of DMEM control; bars, SD. Statistical analysis to DMEM control is calculated using Student's *t* test. *, $P<0.05$; ***, $P<0.001$. (Contributed by Dr. Jurvansuu Jaana)

2.3.4 Overexpression of TMEM18 enhances the *in vitro* glioma-specific migration ability of primary mouse neural stem cells

The function of overexpressing TMEM18 in regulating the glioma tropism of NSCs was further investigated in primary mouse neural stem cells. In Boyden chamber assay, significantly higher migration capacity to U87 and H4 has been achieved in TMEM18-overexpressing primary NSCs, which also displaying migration advantage over control 293FT cells (Figure 2.6).

Primary NSCs/NPs isolated from E14 mouse forebrains are a heterogenous population with multipotency, each of subpopulations capable of giving rise to specific type of neural cells. To understand whether TMEM overexpression has any specific effect on the migration of a particular subtype of NSCs/NPs, we examined the differentiation ability of the primary cells, migrating TMEM18-overexpressing NSCs/NPs collected on the bottom of the insert membrane of the Boyden chamber, and non-migrating cells on the upper side of the membrane. Although there was no obvious difference in cell type distribution between the cells derived from primary NSCs/NPs and the cells from non-migrating TMEM18-overexpressing NSCs/NPs, the cells derived from migrating TMEM18-overexpressing NSCs/NPs displayed morphological features typical of astrocytes and were strongly positive for GFAP, a marker for astroglial precursors and astrocytes (Figure 2.7). The cells derived from migrating NSCs/NPs were negative for O4, a marker for oligodendrocytes, and only weakly stained with anti-β-III-tubulin, a marker for neurons. These

results indicate that glioma-tracking populations of TMEM18-overexpressing NSCs/NPs comprise largely of astrocytic precursors, which is consistent with previous observations from an *in vivo* study reporting that the majority of NSCs that migrated along with glioma outgrowths and satellites were astrocytic precursors (Ehtesham *et al*, 2004).

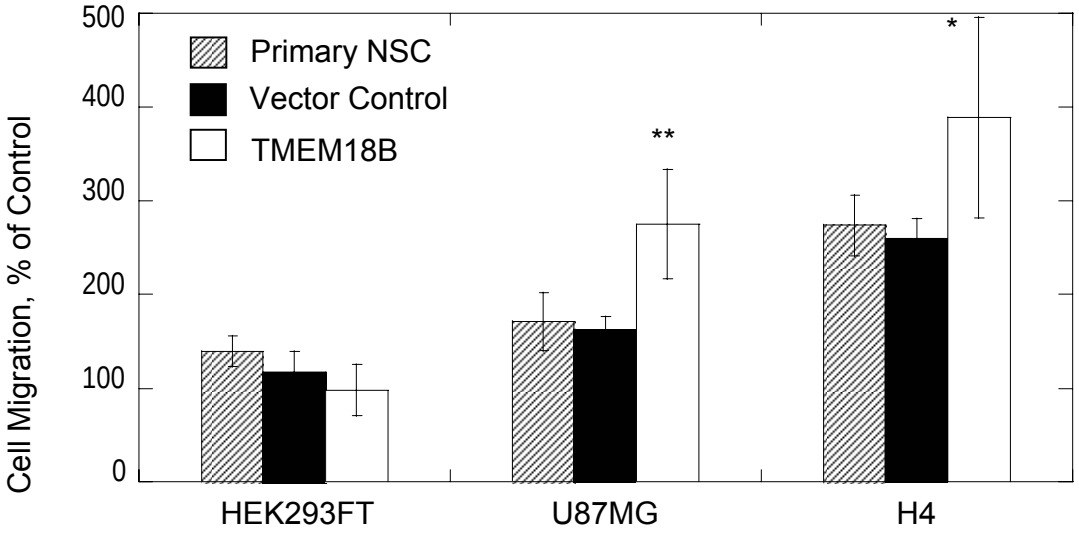


Figure 2.6. TMEM18 overexpression increases the migration activity of primary NSCs in Boyden chamber. Columns, percentage of DMEM control; bars, SD. Statistical comparisons are calculated between cells overexpressing TMEM18 and vector controls using Student's *t* test. *, $P<0.05$; **, $P<0.01$.

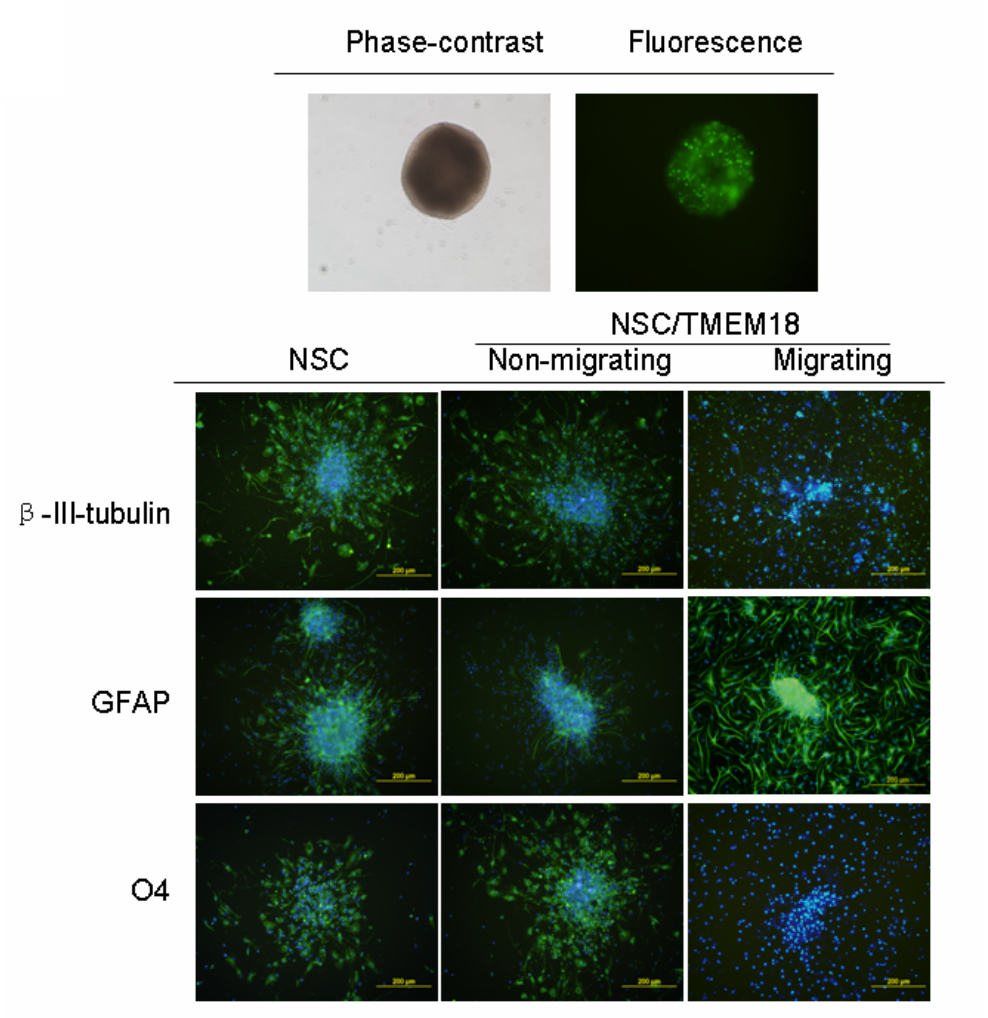


Figure 2.7. Glioma-tracking cells of TMEM18-overexpressing NSCs/NPs in Boyden chamber assay are mainly astrocytic precursors. The upper panels: phase-contrast and fluorescence images show the transfection efficiency of control plasmid pLenti/V5-eGFP. The lower panels: immunostaining using antibodies against β-tubulin, GFAP and O4 (green). DAPI (blue) was used to counterstain nuclei. Note that the cells derived from migrating TMEM18-overexpressing NSCs/NPs were stained strongly with anit-GFAP.

2.3.5 Overexpression of TMEM18 enhances the glioma-directed migration C17.2 in rat C6 glioma models

Encouraged by the above *in vitro* results, we moved on to test whether overexpression of TMEM18 would improve the migration of C17.2 murine neural stem cells toward gliomas in the brain. In a rat C6 glioma xenograft model, green fluorescent dye-labeled TMEM18-overexpressing C17.2 cells were injected together with red fluorescent dye-labeled vector control C17.2 cells on the side of the brain contralateral to the tumor inoculation site. Three weeks after the injection, the brain samples were collected for examination. As shown in Figure 2.8, the labeled green and red cells migrated together (exhibited a yellow color in Figure 2.8 C) toward the tumor side and about half of them had already crossed the middle line of the brain by Week 3. At the front of the migrating cells, many cells with green fluorescence only were observed. The brain sections around the front region were evaluated, where approximately 60% of the migrating cells were stained with the green fluorescent dye and 40% labeled with the red fluorescent dye. These findings suggest an improved migration of the TMEM18-overexpressing cells toward gliomas in the brain.

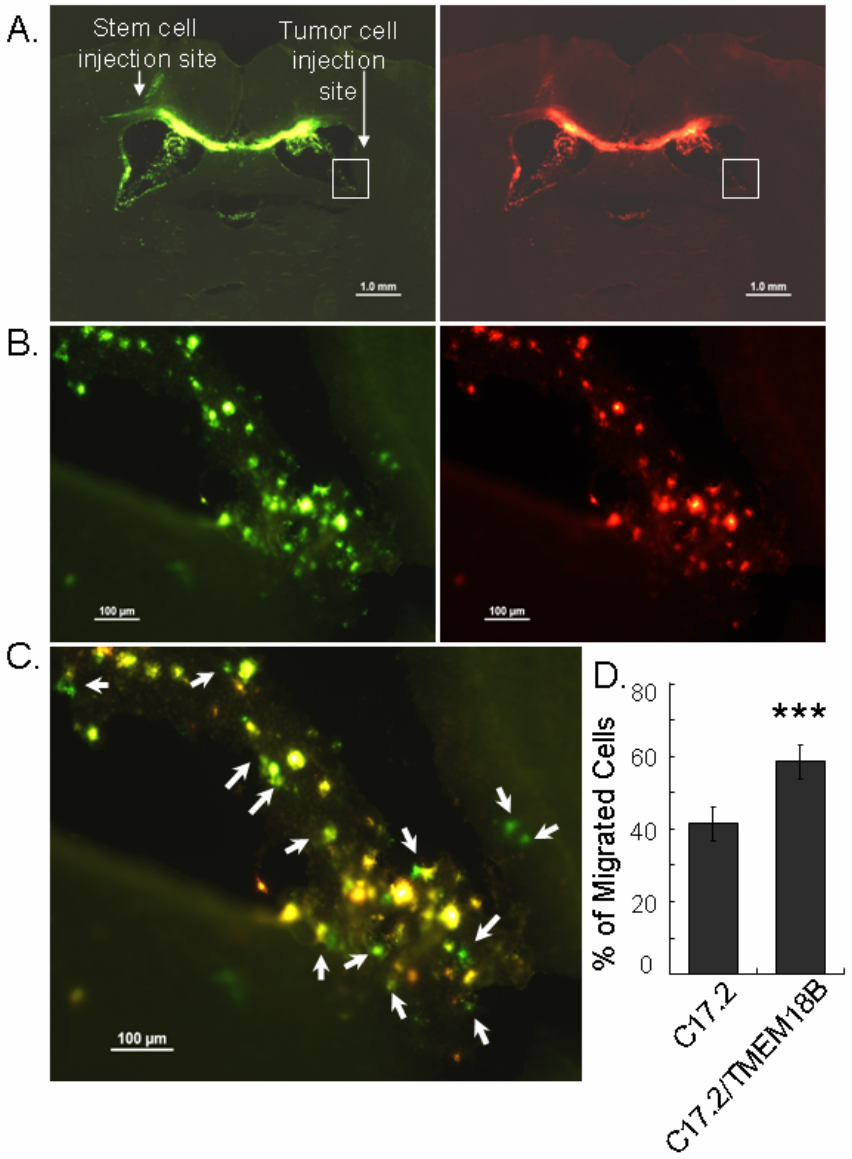


Figure 2.8. TMEM18 overexpression increases the migration of C17.2 neural stem cells toward C6 glioma cells in the rat brain. TMEM18-overexpressing C17.2 cells were labeled with green fluorescence dye-labeled and vector control C17.2 cells were labeled with red fluoresce dye. The squares on the right side in A indicate the front of cell migration toward the tumor, which were shown in a high magnification in B. C is the merged picture of A and B. Dots with yellow color present green and red fluoresce dye-labeled cells moving together. Note many of green fluorescence dye-labeled TMEM18-overexpressing C17.2 cells (arrows) migrating alone. D. Quantification of fluorescence dye-labeled C17.2 (red) and C17.2/TMEM18B (green) cells in the front of cell migration toward the C6 glioma inoculation site. Three stars (***) denote a p-value less than 0.001.

2.3.6 Endogenous TMEM18 is critical for the migration of neural stem/precursor cells

To determine the effect of endogenous TMEM18 in regulating neural stem cell migration, we used RNA interference approach to reduce the expression of TMEM18 in NT2 cells. Puromycin resistant siRNA expression vector was constructed to express siRNA sequences against *TMEM18* and against the *luciferase* gene (as a siRNA control). Two different transductions with two siRNA constructs against *TMEM18* in NT2 cells yielded four populations with different levels of reduction of TMEM18 mRNA expression, ranging from 31, 35, 37 to 65% of the original endogenous TMEM18 mRNA level (Figure 2.9 A). In a 24 hour Boyden chamber assay, siRNAs against *TMEM18* displayed strong inhibitory effects on the migration of NT2 cells when compared with cells transfected with plasmids expressing siRNA against the *luciferase* gene (Figure 2.9 B). Reduction in the amount of *TMEM18* mRNA to 60% of the normal levels lowered the number of cells migrating towards glioma cells to about 50% of the control. Further reduction of *TMEM18* mRNA expression to 31% of the normal level in NT2 cells almost abolished the cell migration ability completely (Figure 2.9 B). Moreover, down-regulation of TMEM18 comparably reduced cell migration toward plain DMEM medium as well (Figure 2.9 B), suggesting that TMEM18 is an important factor regulating general cell motility. The effect of endogenous TMEM18 on the movement was also studied in human embryonic stem (hES) cell-derived neural progenitor cells. Along with the differentiation of hES cells into embryoid body and neural sphere,

TMEM18 mRNA expression increased (Figure 2.10 A). This increase was accompanied with an enhanced migration of cells in neural sphere towards glioma U87MG cells (Figure 2.10 B). Taken together, these findings suggest that the expression of endogenous TMEM18 at a physiological level is crucial to the migration capacity of neural progenitor/stem cells.

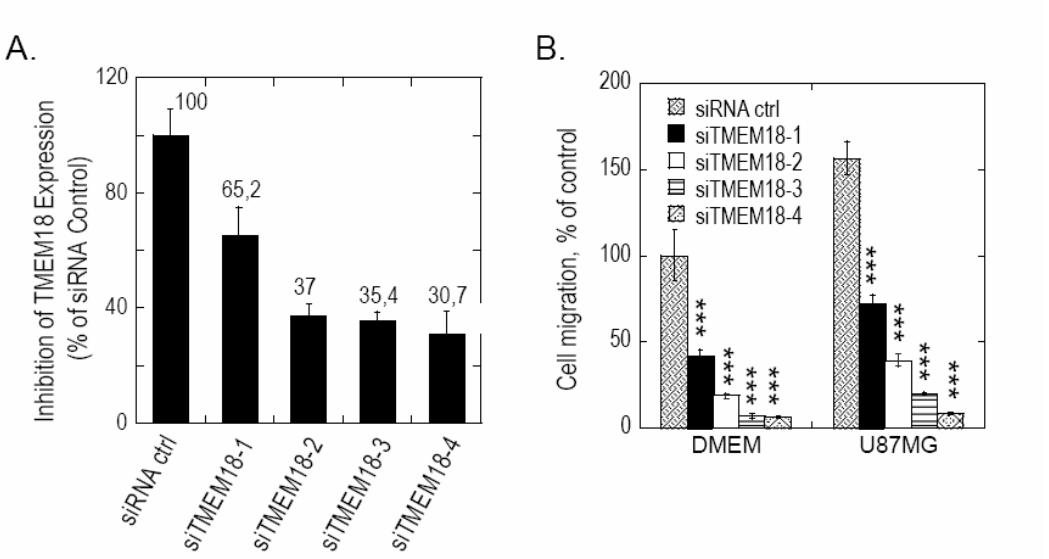


Figure 2.9. Endogenous TMEM18 expression affects cell migration. **(A)** Short interfering RNAs against TMEM18 transcripts reduced the expression of *TMEM18* transcripts in NT2 cells, as quantified by real-time PCR. Two different sequences of short interfering RNAs against *TMEM18* were tested in NT2 cells and yielded four different knock-down levels of the *TMEM18* mRNA. siRNA against luciferase was used as a control. **(B)** Silencing endogenous TMEM18 expression reduced the migration activity of neural stem/precursor cells towards both DMEM and U87MG. (Contributed by Dr. Jurvansuu Jaana)

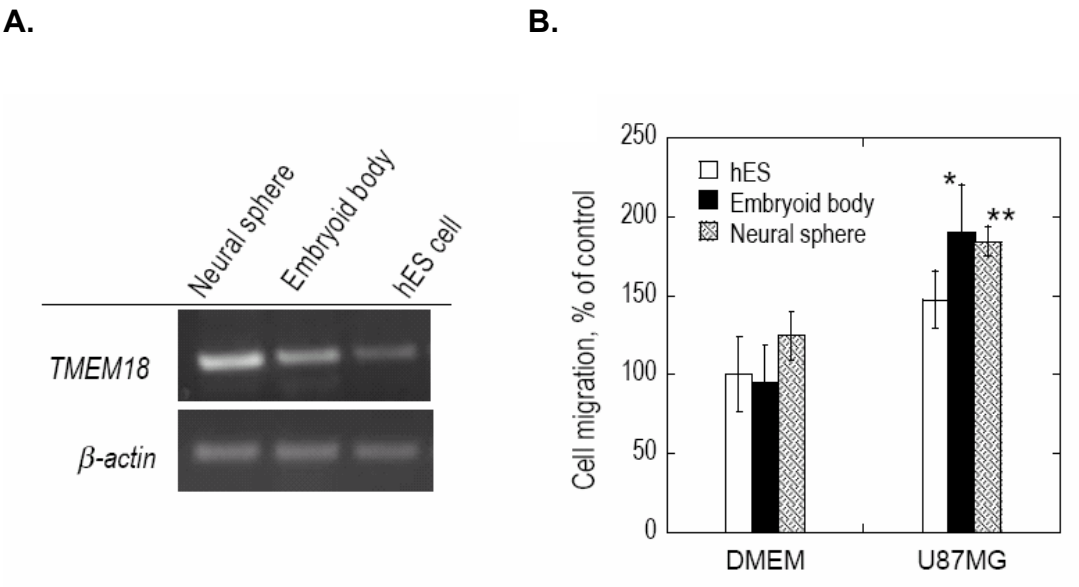


Figure 2.10. Endogenous TMEM18 expression affects cell migration during the differentiation of hES cells. Neural differentiation of hES cells was accompanied by progressive increase in *TMEM18* mRNA expression and cell migration toward glioma cells. Results are presented as percentage of control and the standard deviation is indicated with error bars. Statistical comparison is calculated between cells with downregulated TMEM18 and controls using students T-test. One star (*), two stars (**) and three stars (***) denote p-values less than 0.05, 0.01 and 0.001, respectively.

2.3.7 Up-regulation of CXCR4 by TMEM18 mediates the glioma-specific migration capacity of neural stem/precursor cells

In view of the importance of the chemokine receptor 4 (CXCR4) that governs the migration of stem cells towards gliomas (reviewed by Kucia *et al*, 2005), we investigated whether TMEM18 would affect its expression in neural stem/precursor cells. We observed that, although weak *CXCR4* expression was visible in parental NT2 and C17.2 cells as well as the vector controls, stable overexpression of TMEM18 appeared to raise its expression levels (Figure 2.11). Using an antibody against CXCR4 to block cell surface CXCR4 receptors on these cells, we further observed that the tropism of TMEM18-overexpressing NT2 and C17.2 cells toward U87MG cells was inhibited drastically in Boyden chamber assays and the number of migrating cells went down to a level close to that of basal cell migration in response to serum-free DMEM medium (Figure 2.12). These results suggest that up-regulation of CXCR4 in TMEM18-overexpressing cells might be one possible mechanism underlying the augmented glioma tropism of these cells.

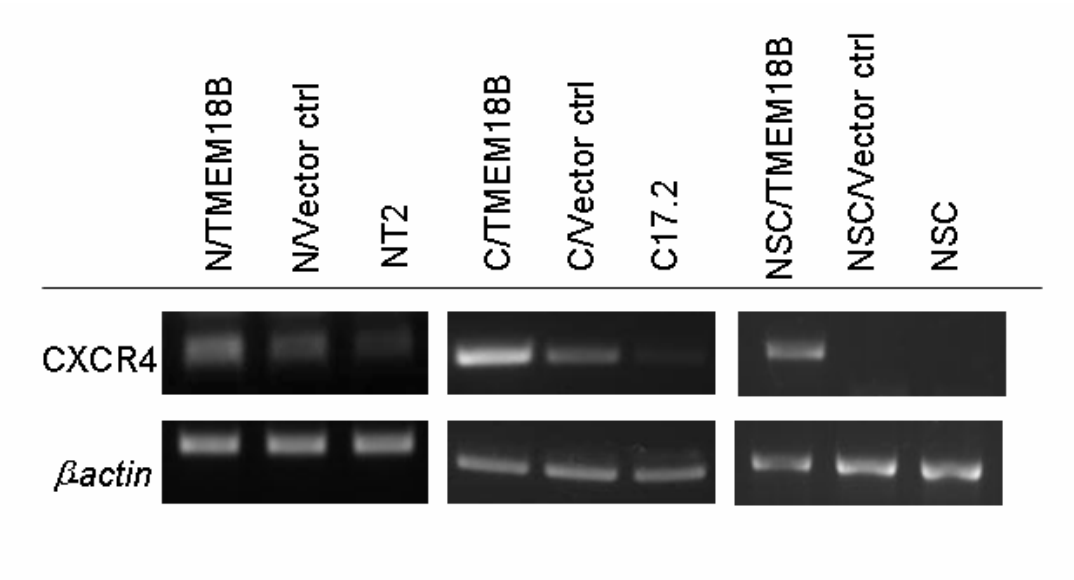


Figure 2.11. RT-PCR demonstrates increased levels of CXCR4 mRNA transcripts in TMEM18-overexpressing NT2 and C17.2 cells.

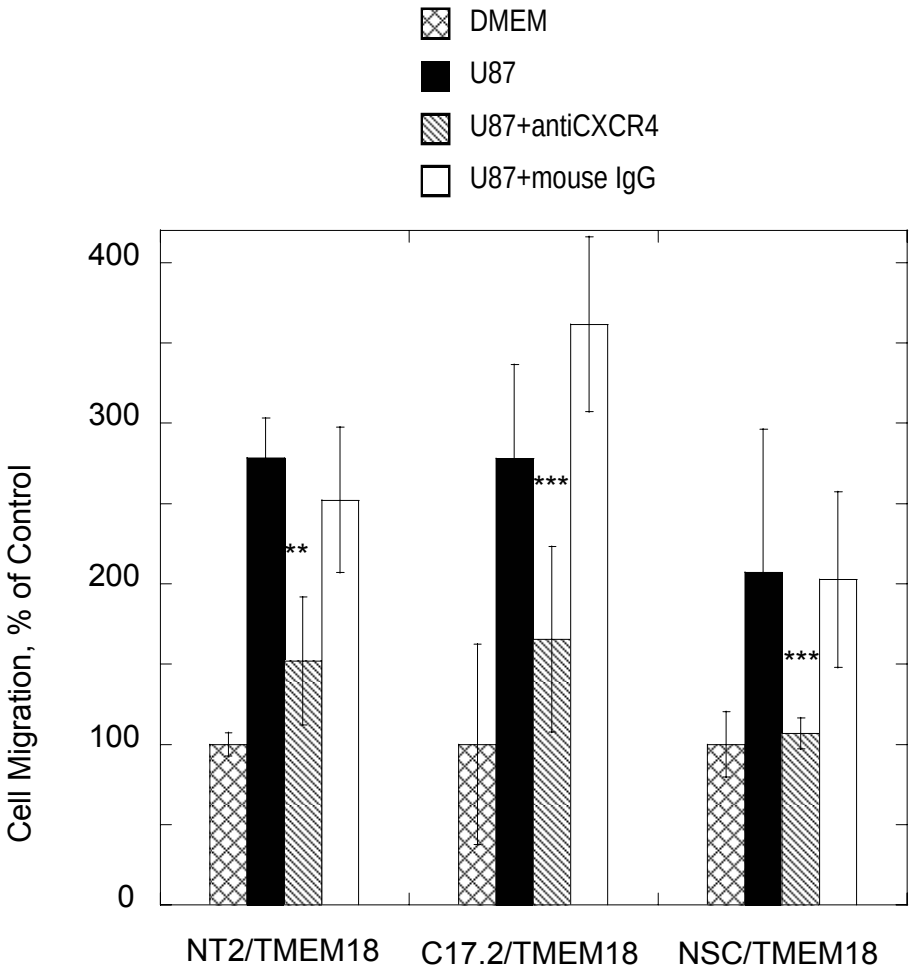


Figure 2.12. Addition of an anti-CXCR4 neutralization antibody significantly decreased neural stem and precursor cell migration toward U87 glioma cells compared to cells treated with nonspecific isotype IgG. Results are presented as percentage of the DMEM control and the standard deviation is indicated with error bars. Two stars (**) and three stars (***) denote p-values less than 0.01 and 0.001 respectively.

2.3.8 The NLS sequence of TMEM18 is sufficient for nuclear targeting

To uncover the cellular localization of TMEM18, a polyclonal antibody against TMEM18 C-terminal peptide was produced. The activity and specificity of the antibody were examined using cellular immunostaining and Western blot analysis (data not shown). In NT2 cells, pre-immunization serum produced almost no signal, whereas the serum against TMEM18 gave a strong immunofluorescence. Western analysis with the serum revealed both endogenous and over-expressed TMEM18 with molecular mass of 18 kDa. The bands disappeared when the serum was incubated in the presence of the immunizing peptide (data not shown).

The TMEM18 antibody stained the cytoplasm of NT2 cells, with Intensive staining in the perinuclear area (Figure 2.13 A & B). In comparison with cytoskeletal structures stained with an alpha-tubulin antibody (Fig 2.13 B, in green), TMEM18 was localized only partly with the areas of the tubulin network (Figure 2.13 B in yellow). Several structures within the nucleus were also positively stained (Figure 2.13 B). With a closer look, the TMEM18 antibody recognized a ring structure superimposed on the nucleus, presumably the nuclear membrane (Figure 2.13 B). This structure became even clearer when the TMEM18 immunofluorescence staining was overlaid with nuclear staining (Fig 2.13 B, in blue).

As TMEM18 contains a putative NLS sequence (Figure 2.3), we tested whether it was effective in directing nuclear location in U87MG cells using a GFP fusion protein approach. Transfection of the control GFP plasmid in U87MG cells led to green fluorescent signals all over the cells, in both the nucleus and the cytoplasm (Figure 2.13 C, GFP). This was expected because the small size of GFP (30 kDa) permits diffusion between the nucleus and the cytoplasm. Noticeably, transfection with a plasmid vector encoding a hybrid protein composed of GFP linked to the putative NLS at the C-terminus of TMEM18 (KED) resulted in significant accumulation of fluorescent signals in the cell nucleus (Figure 2.13 C, GFP-KED). The TMEM18 KED peptide appeared as effective as Tat peptide (Figure 2.13 C, GFP-TAT), a well-established nuclear localization signal peptide, in directing GFP into the nucleus. In addition, diffuse fluorescence signals were detectable in these U87MG cells transfected with the fusion genes.

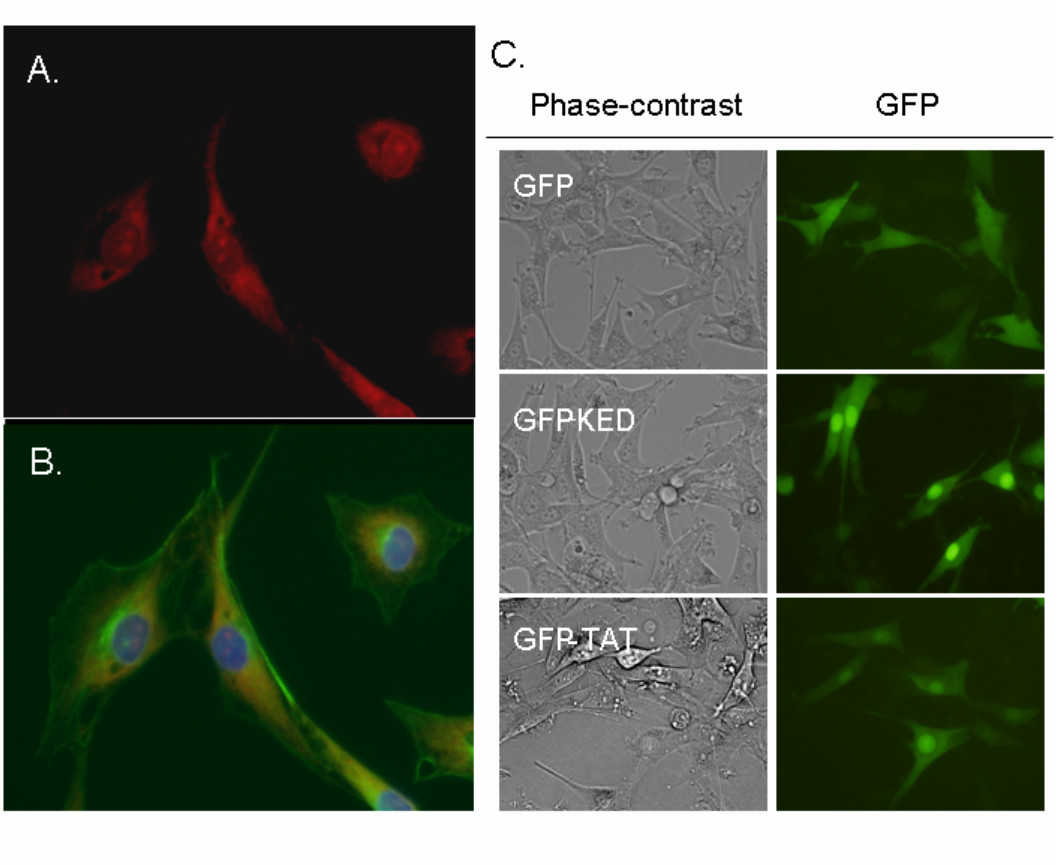


Figure 2.13. Cellular localization of TMEM18 and the function of its NLS. **(A, B)** Cellular localization of TMEM18 protein. NT2 cells were stained against TMEM18 (A & B, red), alpha-tubulin (B, green) and DNA (B, blue). A superimposition of TMEM18, DNA, and alpha-tubulin is shown in (B). The immunostaing reveals the nuclear membrane (a ring structure around the nucleus in A and B) recognized by the TMEM18 antibody. **(C)** GFP fusion protein with N-terminus of TMEM18 is localized to the nucleus. U87 cells were transfected with plasmid vectors expressing GFP, TMEM18 N-terminus-linked GFP (GFP-KED), or HIV TAT-linked GFP (GFP-TAT). Light microscope pictures on left side and fluorescence microscope pictures on right of the same cells. (Contributed by Dr. Jurvansuu Jaana)

2.4 Discussion

We have described in this report the identification and characterization of a novel cell motility modulator TMEM18. TMEM18 was first identified in a screen to discover membrane spanning proteins (Hofmann and Stoffel, 1993). There have been no other publications on this protein since. The functional importance of this protein is highlighted in the strong amino acid conservation throughout mammalians and even reaching to lower forms of multicellular eukaryotes. Furthermore, according to the profile of TMEM18 expressed sequence tags, the protein is transcribed in embryonic developmental states and in many of the adult human tissues (NCBI's EST expression profile viewer). Clearly TMEM18 has a crucial biological function. Based on our results, this function would probably be linked to cell mobility. In particular with respect to tumor therapy, TMEM18 can be used as a specific enhancer for glioma-directed migration of neural stem cells.

The procedure of expression cloning from a cDNA library used in the current study could in theory select the cells that displayed an enhanced ability to migrate specifically to glioma cells, improved nonspecific cell movement, and/or enhanced proliferation rate. The last possibility appears to be invalid, as TMEM18-overexpressing cells had a cell proliferation profile similar to the controls (our unpublished observation). When nonspecific cell movement is taken into consideration, the results that TMEM18-overexpressing cells and control cells migrated at the same rate in plain cell culture medium or toward

non-glioma cells (NIH3T3 mouse fibroblast and 293T human embryonic kidney cells) suggest that TMEM18 overexpression provided no beneficial effects on the general movement of neural stem cells. However, knockdown of endogenous TMEM18 expression with RNA interference had enormous inhibitory effects on the overall movement of neural stem cells. Likewise, along with the increase of TMEM18 expression from an undetectable level to an easily detectable level when human embryonic stem cells differentiated into neural precursor cells, these neural stem/precursor cells displayed an increased capacity of cell migration (Fig. 5E & F). These findings indicate a crucial role of the basal, physiological level expression of TMEM18 for cell movement, which is well consistent with the highly conserved and ubiquitously expressed pattern of TMEM18.

Most interestingly, TMEM18 overexpressing cells respond strongly to glioma cell-secreted cues in both *in vitro* transwell assays and an *in vivo* migration experiment. In transwell assays, cells will have to force themselves through holes in Boyden chamber membrane that are smaller than the normal size of a cell body. It could be even more difficult for cells to migrate to a target site in the brain where cell migration needs to overcome numerous extracellular interactions. We thus conclude that TMEM18 overexpression increases the sensitivity of neural stem cells to appropriate signals that stimulate cell migration. In other words, without appropriate cues, TMEM18 overexpression will have undetectable effects on the movement of neural stem cells.

TMEM18 is expected to be a transmembrane protein. But it did not seem to be located to outer cell membrane, as would be expected for example for chemokine receptors, nor was it spread to cover cytoskeletal structures that would affect cell movement directly. TMEM18 is predicated to possess a NLS sequence at the C-terminus. NLS sequences occur in a subset of soluble nuclear proteins that are imported into the cell nucleus by transport receptors. Membrane proteins with NLS-like sequences are found in the majority of mammalian inner nuclear membrane (INM) proteins (Horton and Nakai, 1997). A recent study in budding yeast demonstrates that NLS sequences are essential for passage of integral membrane proteins through the nuclear pore complex and receptor-mediated transport of the proteins to the INM (King *et al*, 2006). Further studies are warranted to assess whether TMEM18 is really located along the inside of the nuclear envelope.

Cell motility is a highly complex process and involves several factors, from sensing of environmental cues, restructuring the cytoskeleton, dynamic regulation of cell attachment and detachment to extracellular matrix, to signaling between all these processes to coordinate the movements. Cell movement in general can be divided into five different states starting from formation of cell membrane protrusion (lamellabodia and filopbodia), establishment of adhesion complexes into the leading end of the cell, cell body contraction to push the cell forward, and finally release of the adhesion

from the rear end of the migrating cell (Larsen *et al*, 2003). Hence, there are many steps downstream of chemoattractant receptor signaling that can affect cell movement and migration. Bioinformatics searches for protein domains did not reveal any informative features related to a possible biochemical function of TMEM18. Our preliminary data on the up-regulation of CXCR4 in TMEM18-overexpressing cells and inhibiting cell migration by antibodies against CXCR4 suggests an enhanced effect of the SDF-1/CXCR4 axis by TMEM18.

Further research is necessary to define the basic mechanisms underlying the effects of endogenous TMEM18 on general cell migration and the effects of over-expressed TMEM18 to enhance cell response to migration stimulating signals. An adequate understanding of these mechanisms could have important implications for effective cellular delivery of therapeutic agents for brain tumor therapy.

CHAPTER 3

TARGETED SUICIDE GENE THERAPY OF MALIGNANT GLIOMAS USING GLIOMA TROPIC HUMAN PRECURSOR CELLS DERIVED FROM NT2 CELLS

3.1 Introduction

The infiltrative nature of glioma has led to the failure of viral and chemical vectors in clinical trials of glioma gene therapy. Because the distribution of these vectors in brain tumors is limited, only the tumor cells surrounding the injection site are transfected; thus, individual cells migrating from the therapeutic area later give rise to secondary tumors. The inherent glioma tropic property of NSCs makes them well suited for glioma therapy and may overcome the limited therapeutic effects of viral and chemical vectors. Researchers have used genetically engineered NSCs to selectively deliver various antitumor gene products to disseminating tumors. Among them, the best established approach is HSVtk/GCV suicide gene therapy, which has been already well characterized in clinical trials of malignant glioma gene therapy (Pulkkanen and Yla-Herttuala, 2005). In the HSVtk/GCV system, the systemic, nontoxic GCV passes through the BBB and is converted to active drug by HSVtk in NSCs. Hence, the phosphorylated GCV (analog of deoxyguanosine) incorporates itself into the replicating DNA, causing chain termination and killing proliferating cells selectively. The phosphorylated GCV can pass through gap junctions between adjacent cells and kill the surrounding actively dividing tumor cells (Mesnil et al, 1996).

To date, one of the major limitations of stem cell–based glioma gene therapy has been the use of fetal-derived NSCs. To overcome the source limitations and the ethical hurdles faced in therapeutic applications, alternative large-

scale sources of NSCs must be identified (Martino and Pluchino, 2006). The human NPC line NT2 may be a promising cell source for *ex vivo* glioma gene therapy. NT2 NPCs, originally isolated from the testis teratocarcinoma, are able to differentiate to neurons and glias *in vitro* and *in vivo* (Bani-Yaghoub et al, 1999; Fenderson et al, 1987; Miyazono et al, 1995; Pleasure and Lee, 1993; Pleasure et al, 1992; Trojanowski et al, 1993). The gene-expression profile change of NT2 cells during neural differentiation is similar to that of neural progenitor cells during neurogenesis (Przyborski et al, 2000). Interestingly, after 4 to 6 weeks of RA treatment, NT2 cells can differentiate to homogeneous functional neurons with high purity (Pleasure et al, 1992; Saporta et al, 2000; Zeller and Strauss, 1995). RA is a derivative of vitamin A and is known to be functional in the development of the vertebrate CNS (Maden, 2002). During the neuron differentiation of NT2 cells, RA exposure plays a key role in directing neuron phenotype differentiation and suppressing tumorigenicity (Newman et al, 2005).

In this study, we derived glioma tropic precursors from human NPC line, NT2, through treatment with RA. We explored the potential application of these cells in targeted suicide gene therapy of malignant gliomas.

3.2 Materials and methods

3.2.1 Cell culture

NT2 (Ntera-2/D1) NPC line (ATCC), U87 MG (ATCC), and H4 (ATCC) glioma cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO™) supplemented with 10% fetal bovine serum (FBS, GIBCO), 1% penicillin-streptomycin (GIBCO), 2 mM glutamine (GIBCO) and 0.1 mM nonessential amino acids (GIBCO). 293FT cell line was purchased from Invitrogen™, maintained in DMEM supplement with 10% FBS containing 500 μg/mL geneticin (GIBCO).

To produce the stable U87 cell clone–expressing luciferase gene, U87-Luc, U87 cells were seeded in a six-well plate at a density of 5×10^5 per well and transfected with pRC-CMV2-luc plasmid using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. One day later, transfected cells were transferred to a 100-mm cell-culture dish and 1 mg/mL geneticin was added to the medium to select the resistant cells. After 1 week's selection, resistant cells were seeded in a 96-well plate at density of 1 cell per well to form colonies. Ten colonies were selected and further expanded, and the luciferase activity was confirmed using a luminometer (Berthold Lumat LB 9507, Bad Wildbad, Germany) with an assay kit from Promega. The U87-luc clone with the highest luciferase activity was chosen and maintained in medium with 1 mg/mL geneticin.

The neural differentiation of NT2 cells was induced as previously described by Pleasure et al(1992). 2×10^6 cells were seeded in a T75 cell-culture flask 1 day before the RA treatment. Cells were differentiated in complete DMEM culture medium containing 10 μM all trans-RA for 1, 2, and 4 weeks. The culture medium with RA was changed every 2 days and the RA stock solution (10 mM all trans-RA in dimethyl sulfoxide [DMSO]) was diluted with culture medium just before use. Following treatment with RA, differentiated cells were maintained in DMEM supplement with 10% FBS.

3.2.2 Lentivirus preparation and genetic engineering

HSVtk gene was cloned from expression vector pORF-HSVtk (InvivoGen) by PCR using primers 5'-CACC ATGGC CTCGT ACCCC GGCCA TC and 5'TCAGT TAGCC TCCCC CATCT CCCGG into pLenti6/v5-TOPO vector (Invitrogen) followed by sequencing to confirm the construction. HSVtk lentiviruses were produced using the ViraPower™ Lentiviral Directional TOPO Expression Kit (Invitrogen). HSVtk lentiviruses were packaged in 293FT cells by cotransfection of the expression vector pLenti6/v5-HSVtk and the packaging plasmids (pLP1, pLP2, and pLP/VSVG). Lentiviral supernatants were harvested 48 hours after transfection and filtered through a 0.45 μm membrane. To concentrate the lentivirus, virus suspension was centrifuged for 2 hours at 50,000 rpm and 4°C. The virus particles were resuspended with DMEM, stored at –80°C, or immediately used in transduction. In genetic engineering of NT2.RA2 migrating cells and NT2 cells, cells were transduced

overnight by HSVtk lentiviruses in 6 μg/mL polybrene (Invitrogen), followed by blasticidin selection at a concentration of 5 μg/mL for 2 weeks.

3.2.3 Reverse transcription-PCR (RT-PCR)

Total RNA was extracted using an RNeasy Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). One microliter of cDNA reaction mix was subjected to PCR amplification using PCR SuperMix (Invitrogen) as recommended by the manual. Reactions were subjected to 30 PCR cycles after denaturation at 94°C for 4 minutes as follows: 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 60 seconds or 2 minutes. An extension step of 72°C for 5 minutes was included. All products were electrophoresised on a 2% agarose gel. The forward and reverse primers and sizes of RT-PCR productions were as follows: c-Kit, 570 bp, 5'-GCCCACAATAGATTGGTATTT-3' (forward) and 5'-AGCATCTTTACAGC GACAGTC-3' (reverse); CXCR4, 558 bp, 5'-CTCTCCAAAGGAAAGCGAGG TGGACAT-3' (forward) and 5'-AGACTGTACACTGTAGGTGCTGAAATCA-3' (reverse); VEGFR1, 512 bp, 5'-GCAAGGTGTGACTTTTGTTC-3' (forward) and 5'-AGGATTTCTTCCCCTGTGTA-3' (reverse); VEGFR2, 438 bp, 5'-ACGCTGACATGTACGGTCTAT-3' (forward) and 5'-GCCAAGCTTGTACCA TGTGAG-3' (reverse); β-actin, 513 bp, 5'-GCCCAGAGCAAGAGAGGCAT-3' (forward) and 5'-GGCCATCTCTTGCTCGAAGT-3' (reverse); HSVtk, 593 bp,

5’-CAATCGCGAACATCTACACCACA-3’ (forward) and 5’-CCGAAA CAGGGTAAATAACGTGTC-3’ (reverse).

3.2.4 *In vitro* migration assay

The directed migration ability of precursor cells was determined by a modified Boyden chamber assay, with the BD Falcon HTS FluoroBlok 96-Multiwell Insert System (8 µm pore size). One day before the migration assays, glioma cells were seeded at a density of 6.4 × 10^4/well in 96-well companion plates (BD Falcon™) and the medium was replaced with 200 µl Opti-MEM® (Invitrogen). Precursor cells were labeled with Calcein-AM (molecular probes). During labeling, cells were incubated with 5 µg/mL Calcein-AM in culture medium for 10 minutes and then washed with culture medium three times, for 10 minutes each time. Labeled cells were starved overnight in Opti-MEM. The next day, 96-multiwell cell-culture inserts were put into the 96-well companion plates. The labeled cells were suspended in Opti-MEM and seeded into multiple inserts at 2.5 × 10^4/insert. The plates and inserts were incubated together for 24 hours at 37°C in 5% CO_2. The fluorescence from the top side (corresponding to nonmigrating cells) and the bottom side (corresponding to migrating cells) of the plates was measured using a microplate reader (GENios™ Pro, Tecan). Fluorescence background was subtracted during calculation. Values were calculated as the percentage of the bottom reading of the total reading. All experiments were conducted in quadruplicate and values expressed as mean ± SD. Statistical analyses were done using

ANOVA analysis or Student's *t* test. To collect the nonmigrating and migrating cells, a six-well insert system (BD Falcon, 8 μm pore size) was used instead of a 96-multiwell insert system. The cell-culture inserts were washed three times with PBS, and migrating cells on the bottom of the insert membrane and nonmigrating cells on the top side of the membrane were dissociated by trypsination.

3.2.5 *In vivo* migration assay

Adult female Balb/c nude mice (weight 20 g; aged 6-8 weeks) were anesthetized with intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). In intracranial glioma models, green fluorescent dye DiO (Invitrogen)–labeled U87 glioma cells (5×10^5 cells in 10 μL PBS) were injected into the right striatum of the mouse brain (anterior-posterior: 0.0 mm, mediolateral: +2.0 mm and dorsoventral: –3.0 mm from bregma and dura) using a 10-μL Hamilton syringe connected with a 30 G needle at a speed of 0.5 μL/min. The needle was allowed to remain in place for another 5 minutes before being slowly withdrawn at the end of each injection. In subcutaneous glioma models, 10^6 green fluorescent dye DiO (Invitrogen)–labeled U87 glioma cells were subcutaneously injected. On day 14, red fluorescent dye CM-DiI–labeled NT2.RA2 migrating cells and NT2 cells (2×10^6 in 200 μL PBS) were tail-vein injected. On day 14, mice were sacrificed by cardiac perfusion with PBS and 4% paraformaldehyde in PBS. The brains were harvested, suspended in 30% sucrose, and embedded in tissue freezing

medium (Jung). Cryostat sections were prepared and observed under fluorescent microscopy. All handling and care of animals was carried out according to the Guidelines on the Care and Use of Animals for Scientific Purposes issued by the National Advisory Committee for Laboratory Animal Research, Singapore. The current study experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Biological Resource Center, the Agency for Science, Technology and Research of Singapore.

3.2.6 *In vitro* bystander effect

To test the cytotoxicity of GCV, 10^3 NT2 cells, NT2-tk cells, NT2.RA2 migrating cells, NT2.RA2 migrating-tk cells, U87 cells, coculture of NT2-tk cells, and U87 cells at a ratio of 1:1, coculture of NT2.RA2 migrating-tk cells and U87 cells at a ratio of 1:1, were seeded in a 96-well cell-culture plate. Cells were cultured in medium containing 0, 0.1, 1, and 10 μg/mL GCV, and the conditional medium was changed every 2 days. The cell numbers were determined by CellTiter 96® AQueous Assay (MTS, Promega) on day 7. Cell viability was calculated as the percent absorbance of cells cultured without GCV. Values from six wells were expressed as mean ± SD and statistical analyses were carried out using Student's *t* test.

3.2.7 *In vivo* bystander effect

U87-luc cells (0.5×10^6 cells in 10 µL PBS) were intracranially injected into the right striatum (anterior-posterior: 0.0 mm; mediolateral: +2.0 mm; dorsoventral: –3.0 mm from bregma and dura) of female Balb/c nude mice as described previously. On day 7, NT2.RA2 migrating cells, NT2-tk cells, and NT2.RA2 migrating-tk cells (n = 4, 10^6 in 10 µL PBS) were injected into the contralateral side of the mouse brain. Animals were intraperitoneally administered 50 mg/mL GCV daily from day 14 to day 32. Tumor growth was monitored by bioluminescent imaging of U87-luc cells with the IVIS Imaging System (Xenogen). Twenty minutes before *in vivo* imaging, isoflurane gas–anesthetized animals were injected intraperitoneally with D-luciferin (Promega) at 100 mg/kg in PBS. The animals were then placed on a warmed stage inside the camera box. The detected light emitted from U87-luc cells was digitized and electronically displayed as a pseudocolor overlay onto a grayscale image of the animal. Images and measurements of luminescent signals were acquired and analyzed using Living Image® software (Xenogen), quantified as photons per second.

3.3 Results

3.3.1 Generation of glioma tropic precursor cells from NT2 cells

3.3.1.1 Retinoid acid treatment induces the neuron differentiation of NT2 cells and improves migration capacity toward U87 cells

NT2 cells were treated with trans-RA for 1, 2, and 4 weeks, and differentiated cells were named NT2.RA1, NT2.RA2, and NT2.RA4, respectively. Figure 3.1 shows the morphology changes after the RA treatment. Some cells formed extremely dense multilayered culture and others formed clumps of neuron-like cells, as described previously by pleasure et al(Pleasure et al, 1992). The longer the RA treatment lasted, the more neuron-like clumps were observed. To assess the effect of RA treatment on cell migration, we used a Boyden chamber migration assay to examine the migration of RA-treated NT2 cells toward human U87MG glioma cells. Blank medium was used as a negative control and 10% FBS was used as a positive control. The glioma tropism of NT2 cells was improved after RA treatment (Figure 3.2). Compared with NT2.RA1 and NT2.RA4 cells, NT2.RA2 cells displayed a high migration capacity toward U87 glioma cells (26%) and simultaneously maintained low unspecific migration toward blank medium MEM (4%). NT2.RA1 and NT2.RA4 cells showed considerable migration toward U87, but much higher unspecific migration toward MEM. These results suggest that, with the effect of RA-induced differentiation, NT2.RA2 cells have the highest glioma-specific migration. Thus, these cells were selected for further investigation.

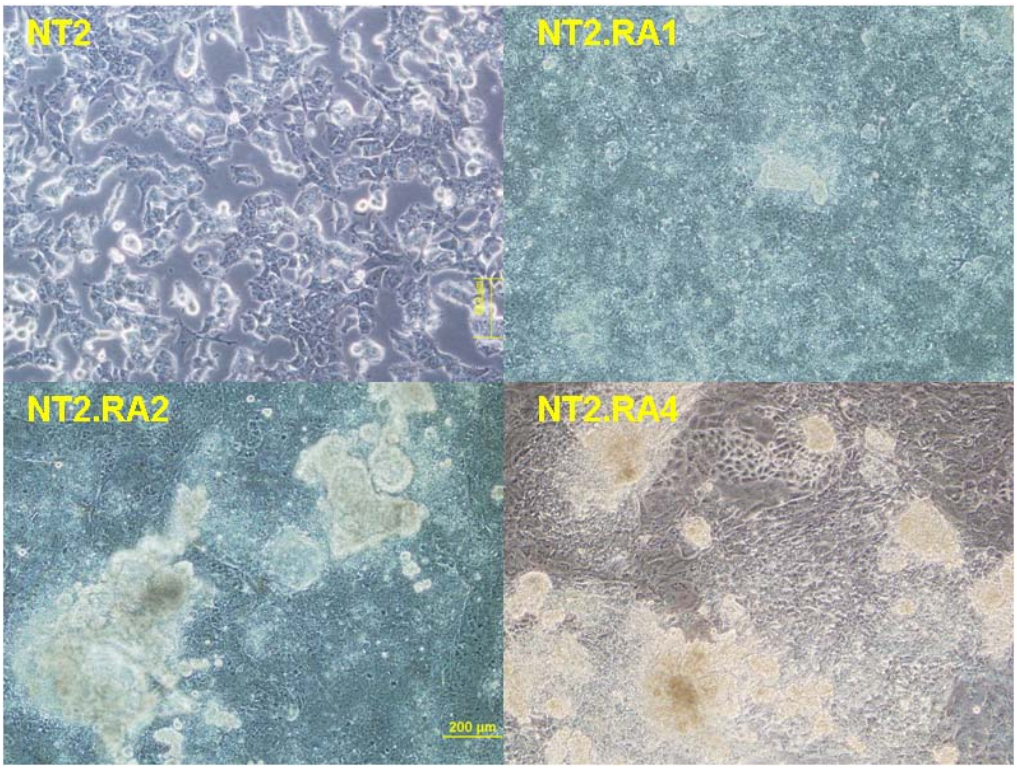


Figure 3.1. RA treatment induces the neural differentiation of NT2 cells. Phase-contrast photographs show the morphologic changes to NT2 cells that occur during RA treatment for 1, 2, and 4 weeks.

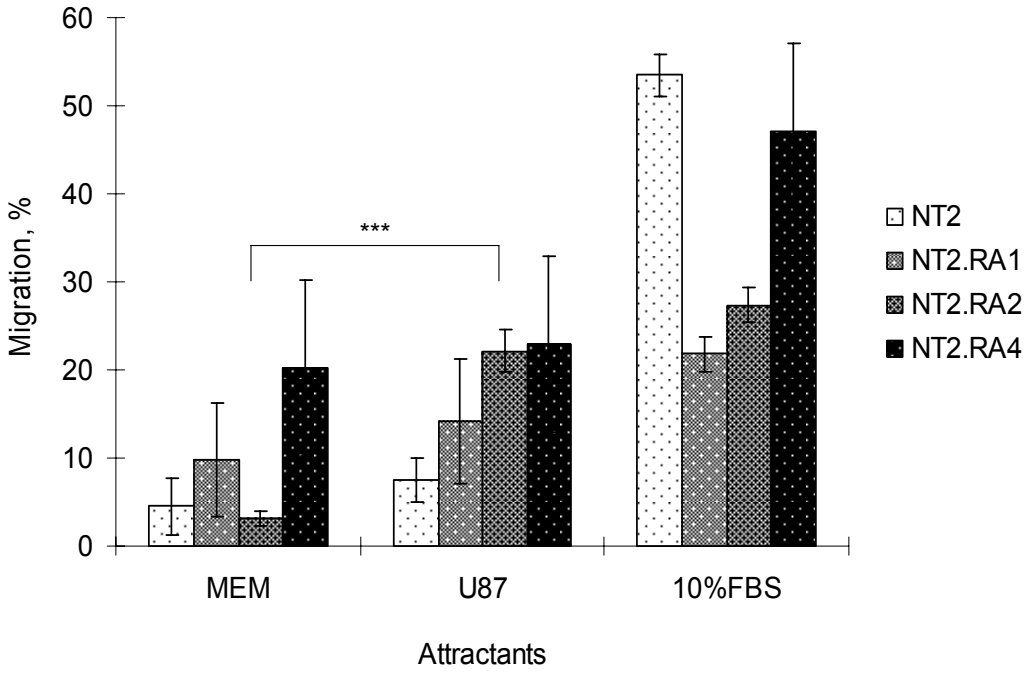


Figure 3.2. RA treatment increases the migration of NT2 cells toward U87 cells in modified Boyden chamber assays. Columns: percentage of fluorescence reading from transmigrating cells in total reading; bars: SD. Statistical analysis calculated using two-factor ANOVA and Student's *t* test. ****P* <0.001.

3.3.1.2 Migration screening selects cells with enhanced glioma-directed migration

To further improve glioma tropism, NT2.RA2 cells went through another transwell migration screening. In the screening, U87 glioma cells were loaded in the lower chamber of the system as attractants. Nonmigrating cells stayed on top of the membrane, whereas cells that were primed to migrate went through 8 μm pores into the opposite site of the transwell insert membrane. Migrating and nonmigrating cells were isolated, and the migration capacity toward U87 glioma cells was tested by modified Boyden chamber migration assays. The results of the migration assays were examined by both fluorescence microscope and microplate reader. Cells were labeled with green fluorescence dye, Calcein-AM so that the number of migrated cells could be observed under the fluorescence microscope (Figure 3.3). After the screening, NT2.RA2 migrating cells showed a higher migration capacity toward U87 cells than did NT2.RA2 nonmigrating cells. The results were also quantified using a TECAN microplate reader (Figure 3.4). The migration percentage of NT2.RA2 migrating cells was 42% and that of NT2.RA2 nonmigrating cells was 15%. The glioma tropism of NT2.RA2 cells was further enhanced by migration screening.

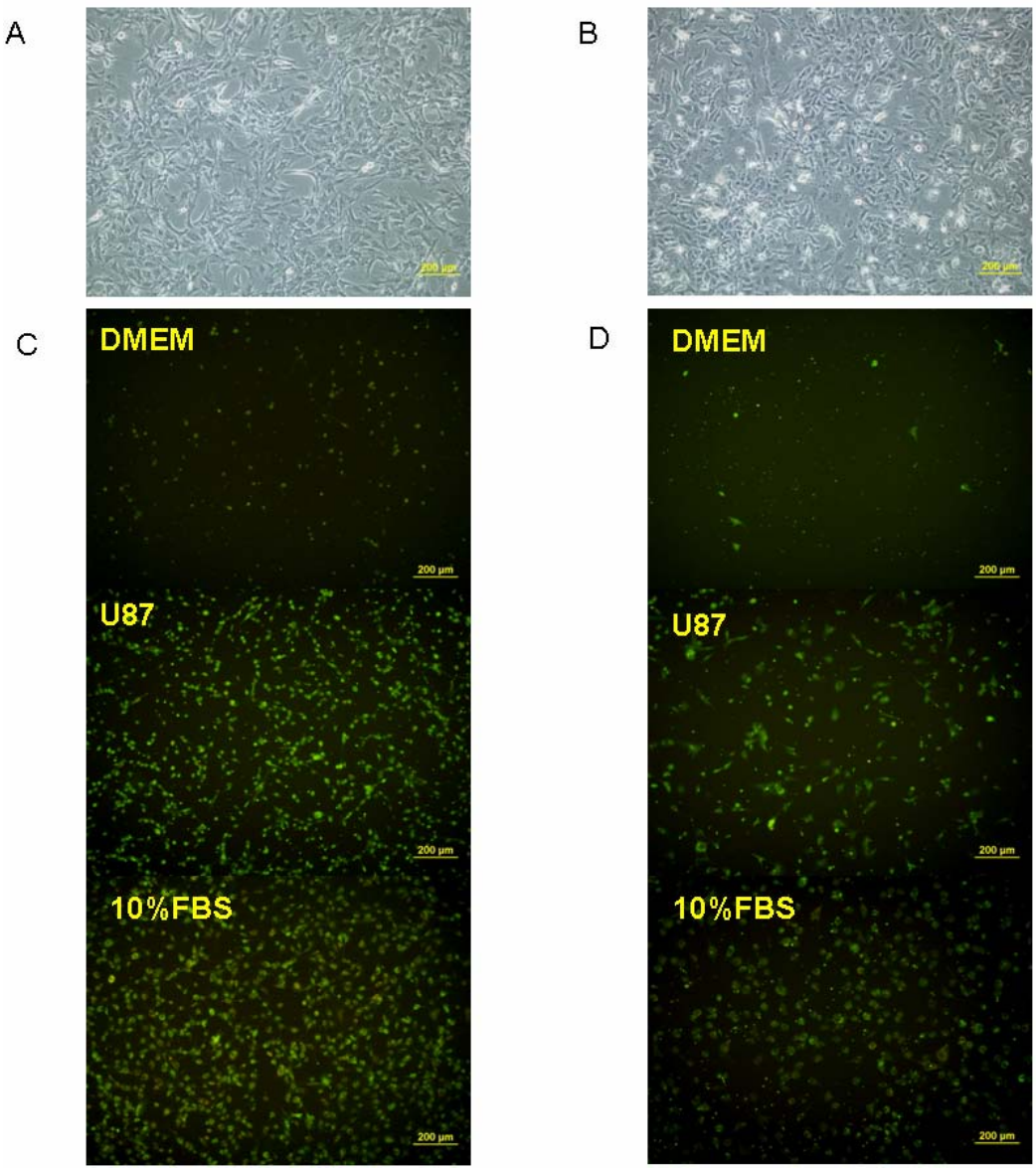


Figure 3.3. NT2.RA2 migrating cells and NT2.RA2 nonmigrating cells after migration screening. Phase-contrast photographs show the morphology of NT2.RA2 migrating cells (A) and NT2.RA2 nonmigrating cells (B). Fluorescence photographs show the Calcein-AM–labeled transmigrated NT2.RA2 migrating cells (C) and NT2.RA2 nonmigrating cells (D) toward MEM, U87, and 10% FBS from the bottom side in Boyden chamber assays.

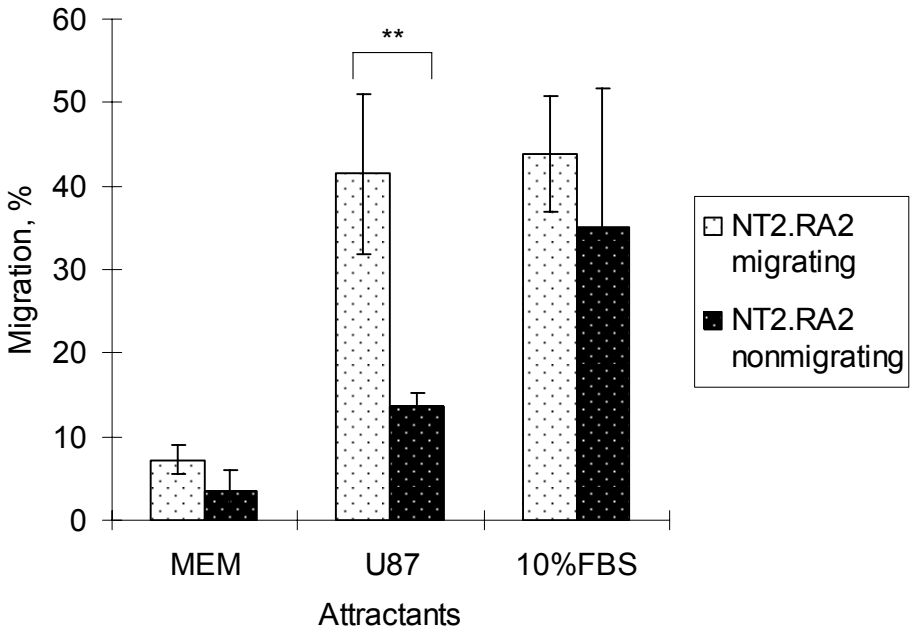


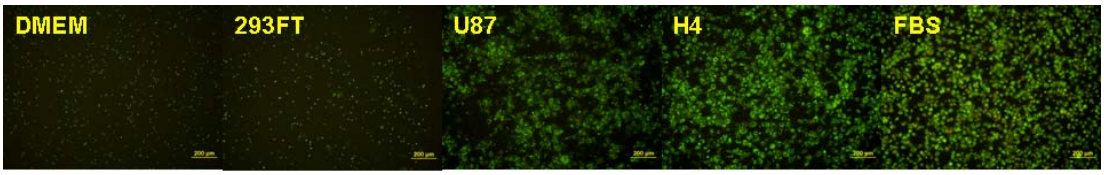
Figure 3.4. Glioma tropism of NT2.RA2 cells is improved by migration screening. *In vitro* migration of NT2.RA2 migrating cells toward U87 cells compared with NT2.RA2 nonmigrating cells; $P < 0.01$. Columns: percentage of bottom reading in total reading; bars: SD.

3.3.2 *In vitro* glioma tropism evaluation of NT2.RA2 migrating cells

3.3.2.1 The enhanced migration capacity of NT2.RA2 migrating cells is glioma specific and endured during long-term culture

We went on to test the migration capacity of NT2.RA2 migrating cells toward more cell lines. Glioma cells lines U87 and H4 were selected to represent glioma cells. Human kidney cell line 293FT served as control. NT2.RA2 migrating cells displayed significantly greater migration capacities when compared with NT2.RA2 nonmigrating cells and NT2 cells (Figure 3.5). These cells responded not only to U87 cells, but also to another glioma cell line, H4, by displaying a significant migration advantage over control 293FT cells. Moreover, the glioma-directed migration almost reached the level of the positive control, 10% FBS. At the same time, NT2.RA2 migrating cells again showed low migration capacities toward nontumor cell lines (ie, human kidney cell line 293FT), similar to plain medium. Hence, the enhanced migration capacity of NT2.RA2 migrating cells is glioma specific. The NT2.RA2 migrating cells maintained long-term proliferation in cell-culture condition. The cells were passaged 36 times and maintained normal cell viability. *In vitro* migration assay showed that glioma-specific tropism remained conserved after 36 passages (Figure 3.6). The migration capacity of NT2.RA2 migrating cells toward glioma cells, U87 and H4, and nonglioma cells, 293FT, demonstrated a similar pattern after long-term subculture. The migration of NT2.RA2 migrating cells toward U87 was 2.5-fold of that of NT2 cells; and toward H4 the value was 6-fold of that of NT2 cells.

A



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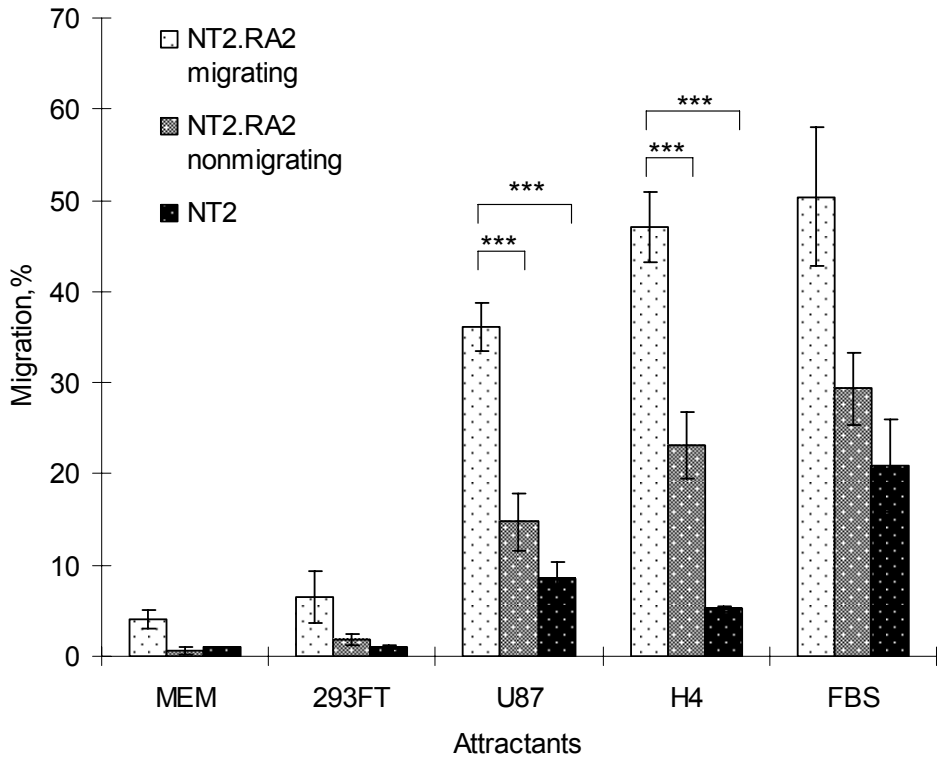

Figure 3.5. Glioma-specific tropism of NT2.RA2 migrating cells. (A) Fluorescence photographs showing the Calcein-AM–labeled transmigrated NT2.RA2 migrating cells, to MEM, 293FT, U87, H4, and 10% FBS from the bottom side of cell-culture inserts. (B) Percentage of transmigrating cells measured by a Tecan microplate reader. Columns: percentage of fluorescence reading from transmigrating cells in total reading; bars: SD. Statistical analysis calculated using Student's *t* test. ****P* <0.001.

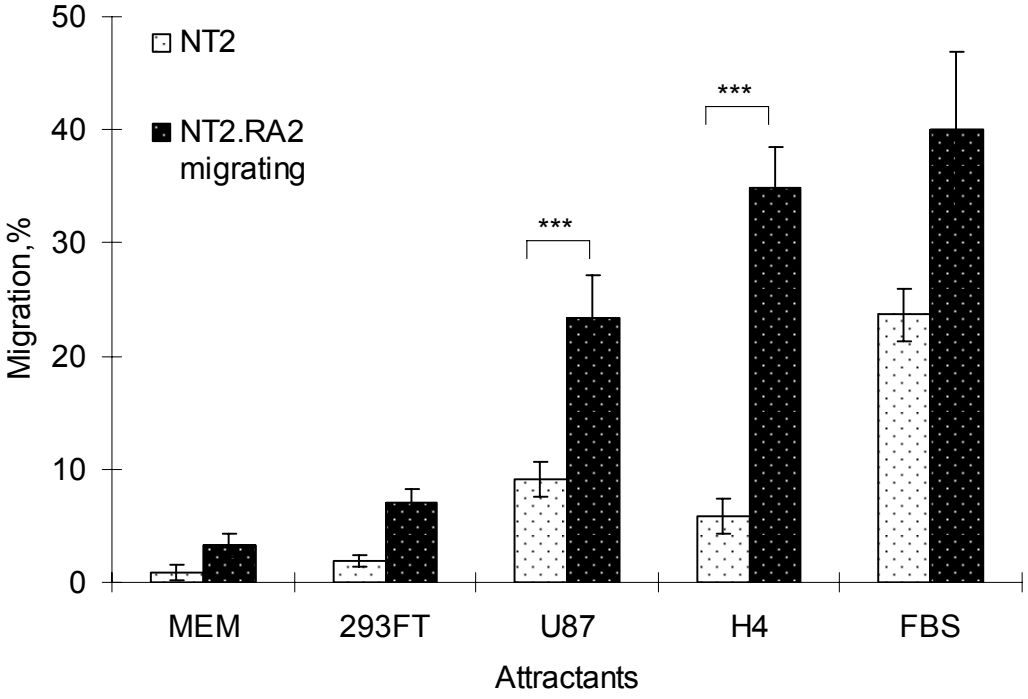

Figure 3.6. Glioma-specific tropic behavior of NT2.RA2 migrating cells is preserved after 36 generations. *In vitro* migration of 36-times-subcultured NT2.RA2 migrating cells and NT2 cells to MEM, 293FT, U87, H4, and 10% FBS was measured by a Tecan microplate reader. Columns: percentage of fluorescence reading from transmigrating cells in total reading; bars: SD. Statistical analysis calculated using Student's *t* test. ***P <0.001.

3.3.2.2 Molecular changes associated with enhanced glioma-specific migration

There have been several instances showing that chemokines and growth factors secreted by tumors regulate the migration of stem cells toward gliomas. We investigated whether enhanced glioma-specific migration of the NT2.RA2 migrating cells could be related to the molecular changes of the chemokine and growth factor receptors. Four factors reported to regulate the NSC migration were selected in the study, including: the chemokine receptor 4 (CXCR4), receptor of stromal cell-derived factor 1α (SDF-1α) (Allport et al, 2004; Ehtesham et al, 2004; Imitola et al, 2004); c-kit, the receptor of stem cell factor (SCF) (Erlandsson et al, 2004; Sun et al, 2004); vascular endothelial growth factor receptor 1 (VEGF1), ; and vascular endothelial growth factor 2 (VEGF2) (Schanzer et al, 2004; Schmidt et al, 2005). We observed that, although weak expression of CXCR4, c-kit, VEGF1, and VEGF2 was visible in NT2 cells and NR2.RA2 nonmigrating cells, the four receptors were all highly expressed in NT2.RA2 migrating cells (Figure 3.7). These results suggest that the enhanced glioma-specific migration of NT2.RA2 migrating cells may be because of the high expression of chemokine and growth factor receptors.

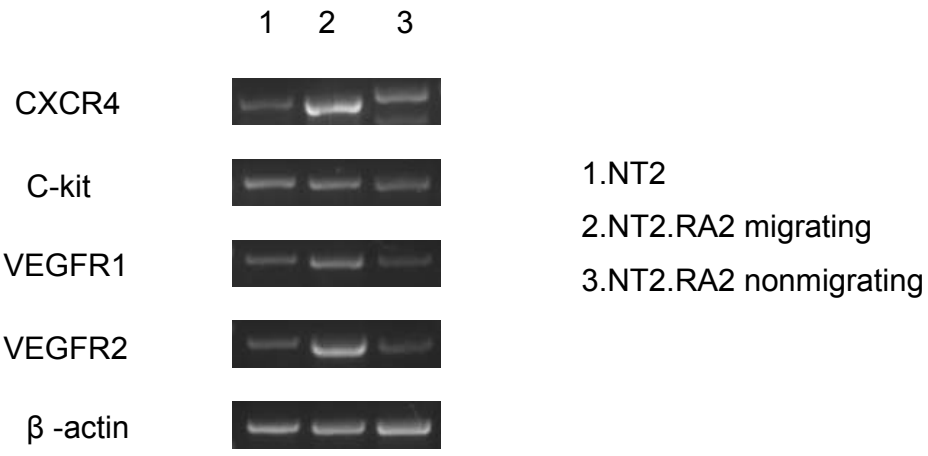

Figure 3.7. The analysis of chemoattractant receptors using RT-PCR. The expression level of CXCR4, C-kit, VEGFR1, and VEGFR2 in NT2, NT2.RA2 migrating, and NT2.RA2 nonmigrating cells was confirmed and visualized on 2% agarose gel.

3.3.3 *In vivo* glioma tropic behavior of NT2.RA2 migrating cells

3.3.3.1 NT2.RA2 migrating cells target subcutaneously implanted U87 gliomas after systemic administration

Nude mice were subcutaneously implanted with U87 gliomas to determine whether NT2.RA2 migrating cells have the capacity to migrate specifically toward tumors *in vivo*. Two weeks later, CM-DiI–labeled NT2.RA2 migrating cells and NT2 cells were injected into the tail vein. After another week, the animals were killed and the tumor sections were processed in order to detect the CM-DiI–labeled NT2.RA2 migrating cells and NT2 cells. The fluorescence images are shown in Figure 3.7. Both NT2.RA2 migrating cells and NT2 cells migrated to the tumor site after intravascular administration. NT2.RA2 migrating cells migrated throughout the entire tumor mass while NT2 cells stayed mainly at the edge of the tumor, suggesting that NT2.RA2 migrating cells could target the subcutaneously implanted U87 gliomas *in vivo*. Glioma tropism is improved by RA treatment and migration screening.

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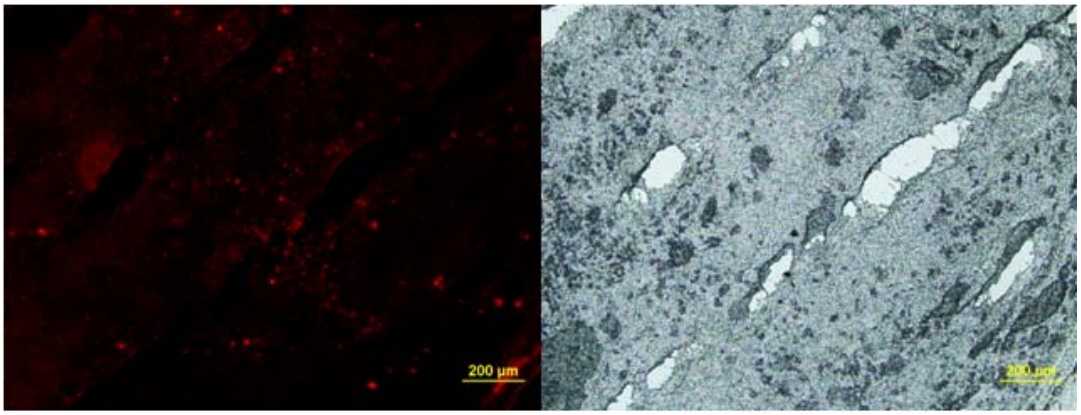

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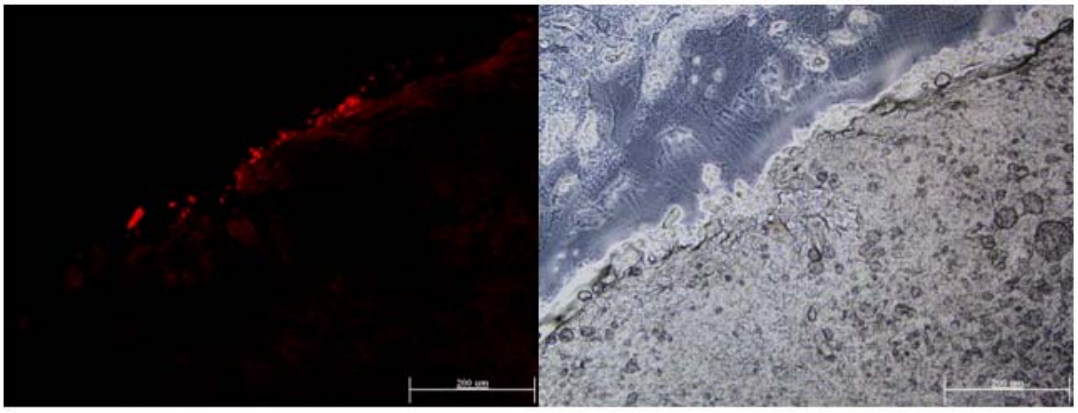

Figure 3.8. After systemic administration, NT2.RA2 migrating cells target the subcutaneously implanted U87 gliomas. Fluorescence photographs show the distribution of CM-DiI–labeled NT2.RA2 migrating cells (A) and NT2 (B) cells in subcutaneously inoculated U87 gliomas. The morphology of tumor sections is presented in bright field images.

3.3.3.2 NT2.RA2 migrating cells target intracranial U87 gliomas after intravenous administration

To determine whether the NT2.RA2 migrating cells could target the intracranial tumors, DiO-labeled U87 gliomas were injected into the striatum of nude mice. After 2 weeks of tumor formation, CM-DiI–labeled NT2.RA2 migrating cells and NT2 cells were injected into the tail vein. One week later, NT2.RA2 migrating cells were distributed throughout the intracranial tumor mass, but were not found in surrounding normal-appearing brain tissue elsewhere in the brain. NT2 cells could also target the tumor, but some stayed in the normal brain region surrounding the tumor. These results indicate that NT2.RA2 migrating cells are potential gene-delivery vectors that, after systemic administration, could target the brain tumors.

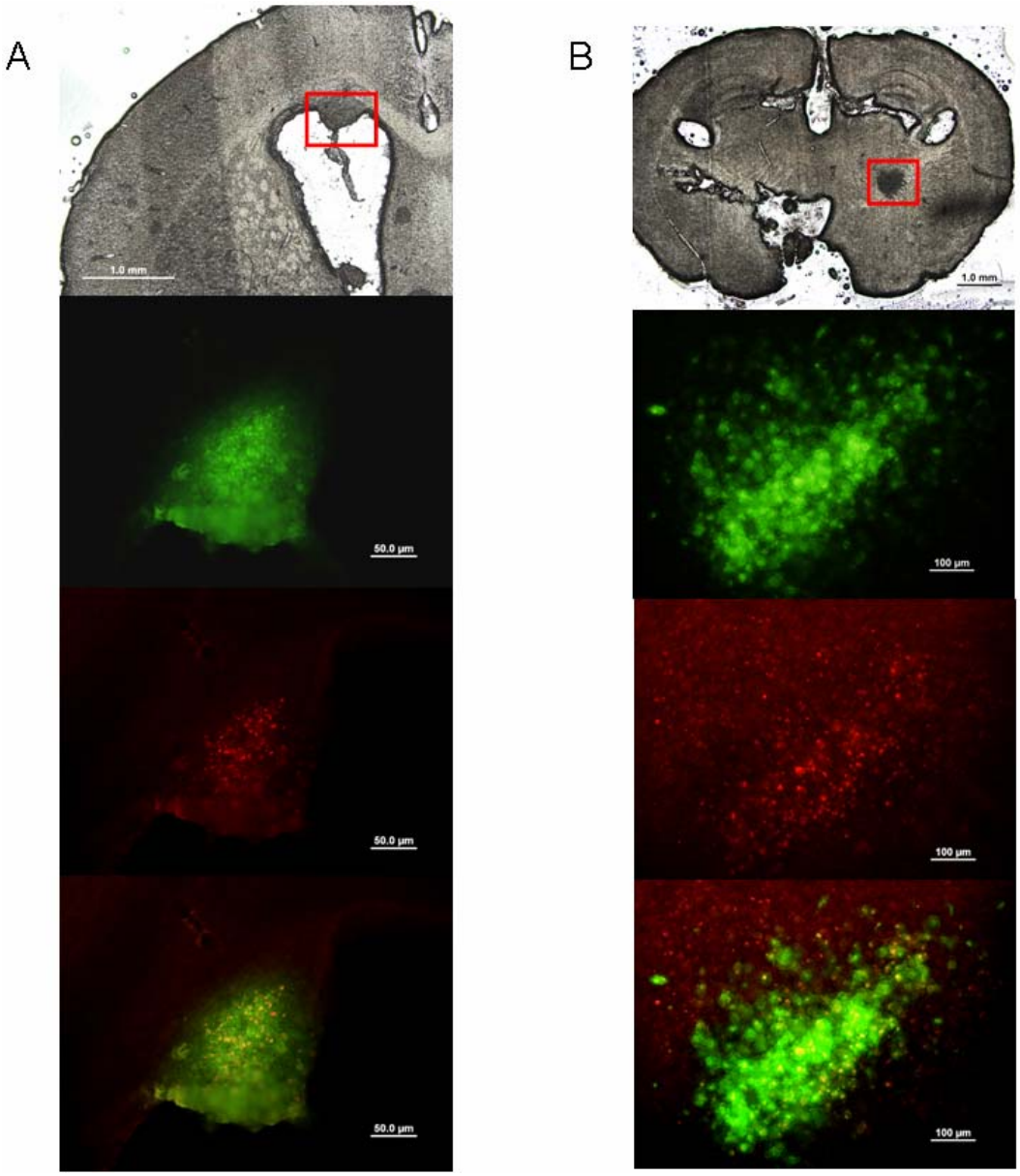

Figure 3.9. After intravascular administration, NT2.RA2 migrating cells target intracranial U87 gliomas. Light microscopy shows U87 tumor formation after intracranial injection. Corresponding high-magnification fluorescent photographs show the distribution of CM-DiI–labeled NT2.RA2 migrating cells (red) (A) and CM-DiI–labeled NT2 cells (red) in DiO-labeled U87 glioma cells (green).

3.3.4 *In vitro* bystander effects mediated by precursor cells transduced with HSVtk gene

3.3.4.1 Transgene expression and sensitivity to GCV

Lentivirus-transduced NT2.RA2 migrating cells and NT2 cells expressing HSVtk transgene, NT2.RA2 migrating-tk, and NT2-tk, were prepared. Expression of HSVtk transcript was confirmed by reverse transcription-PCR (Figure 3.10). The HSVtk transcript was found to be expressed in NT2.RA2 migrating-tk and NT2-tk cells, but not in the parental RA2 migrating and NT2 cells.

To test sensitivity to GCV, NT2, NT2-tk, NT2.RA2 migrating and NT2.RA2 migrating-tk cells were cultured for 7 days with various concentrations of GCV (0.1-10 μg/mL). The results of MTS assays are shown in Figure 3.11. Cells cultured in medium without GCV were used as controls. The cytotoxicity of GCV to NT2-tk and NT2.RA2 migrating-tk cells was observed at a concentration as low as 0.1 μg/mL; about 80% of cells were killed by phosphorylated GCV. The cell number was decreased with the increase of GCV concentration; only about 10% survived at a concentration of 10 μg/mL. Interestingly, NT2.RA2 migrating-tk cells are more resistant to GCV than NT2-tk cells, at low concentrations, 0.1 μg/mL and 1 μg/mL of GCV. In contrast, no significant toxicity was observed in parental NT2 and NT2.RA2 migrating cells. RA treatment may also enhance the resistance of precursor cells to toxins.

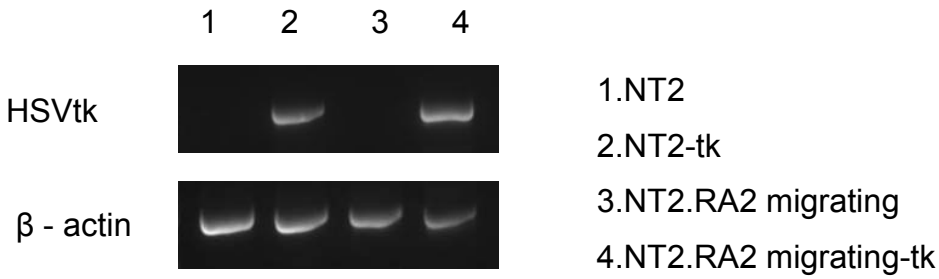

Figure 3.10. The analysis of HSVtk expression using reverse transcription-PCR.

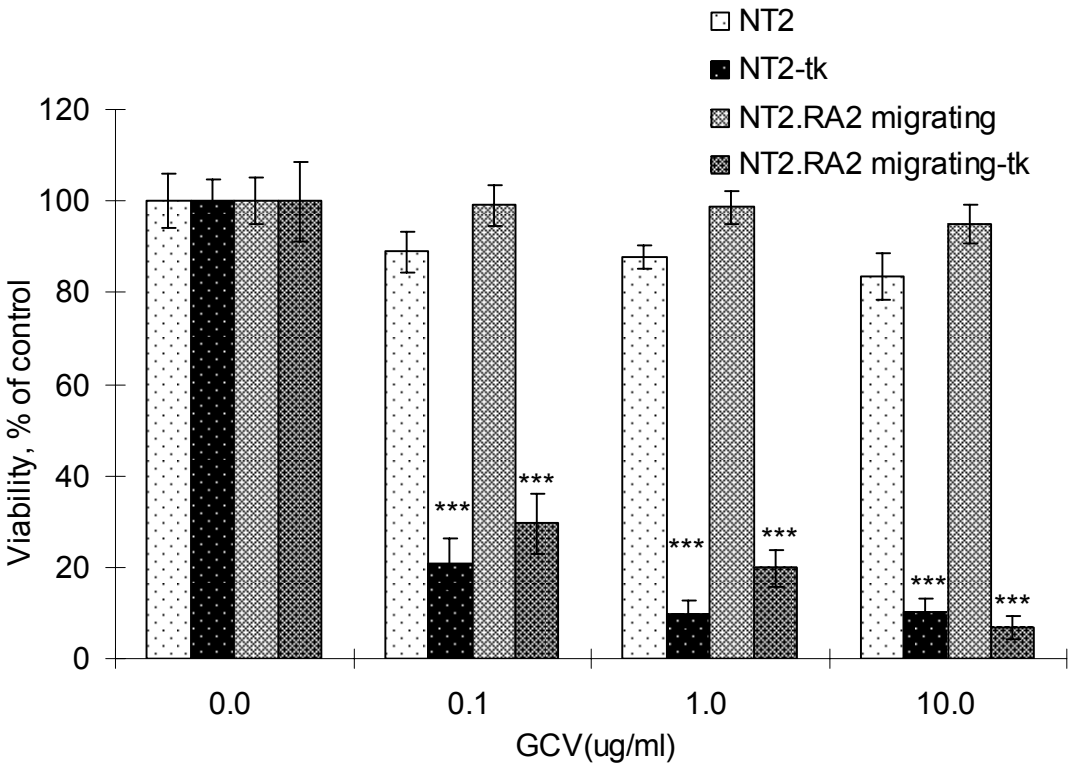

Figure 3.11. *In vitro* sensitivity to GCV evaluated by MTS assay (6 repeats). Columns: mean cell viability (percentage of control cells cultured without GCV); bars: SD. Statistical comparisons to controls calculated using two-factor ANOVA and Student's *t* test. ****P* <0.001.

33.3.4.2 *In vitro* therapeutic efficacy

To examine the bystander effect of phosphorylated GCV released from tk precursor cells, cell viability studies were done in a coculture system (Figure 3.12). The GCV-containing medium alone showed quite low toxicity to U87 cells. When U87 cells were cocultured with NT2-tk and NT2.RA2 migrating-tk cells at the ratio of 1:1, cell proliferation was significantly inhibited by the phosphorylated GCV released from tk precursor cells, and at a GCV concentration of 10 μg/mL, 80% of cells were killed. It is worth noting that, at low concentrations of GCV (0.1 μg/mL and 1 μg/mL), NT2.RA2 migrating-tk cells showed a stronger tumor-inhibiting effect than NT2-tk cells. This phenomenon could be explained by previous findings that NT2.RA2 migrating-tk cells are more resistant than NT2-tk cells are to GCV. These results indicate that NT2.RA2 migrating-tk cells can convert sufficient amounts of GCV to effectively kill U87 cells *in vitro*.

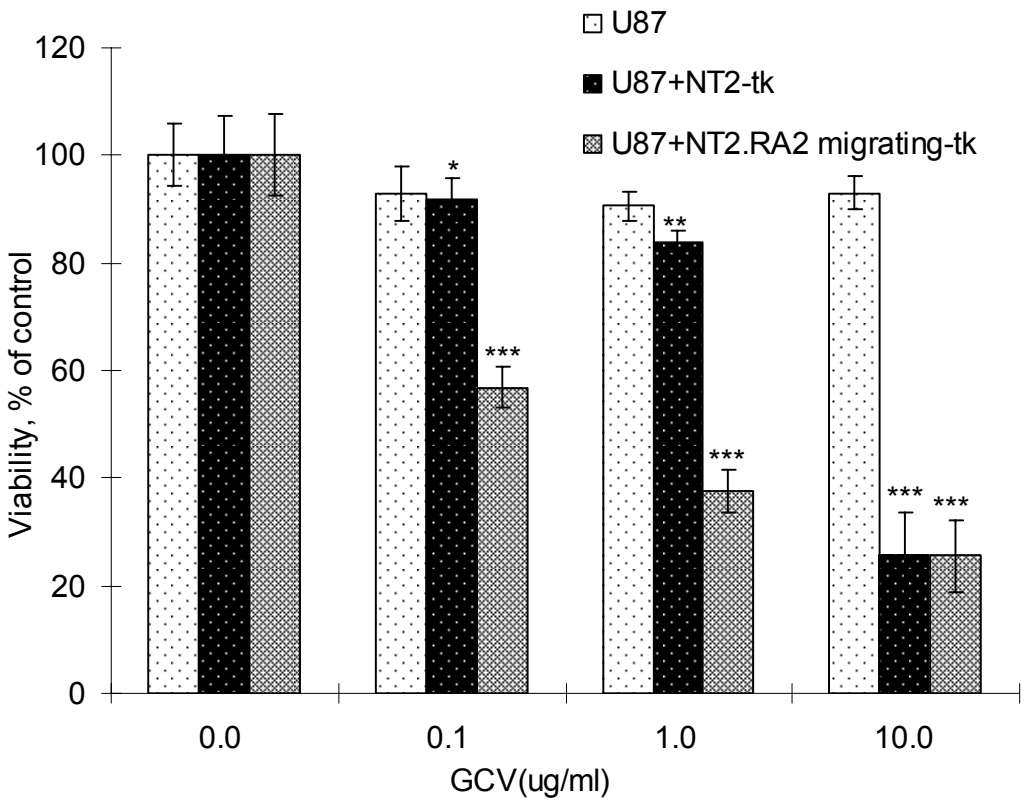

Figure 3.12. *In vitro* therapeutic efficacy of NT2.RA2 migrating-tk cells in the coculture system (6 repeats). Columns: mean cell viability (percentage of control cells cultured without GCV); bars: SD. Statistical comparisons to controls calculated using two-factor ANOVA and Student's *t* test. **P* <0.05; ***P* <0.01; ****P* <0.001.

3.3.5 *In vivo* therapeutic effect of HSVtk precursor cells

To assess antitumor efficacy in animal models with glioma, we intracranially injected NT2.RA2 migrating-tk cells into nude mice bearing tumors (Figure 3.13). 0.5×10^6 U87-luc cells were inoculated into the right striatum of the brain. Seven days later, 10^6 NT2.RA2 migrating-tk, NT2-tk, and NT2.RA2 migrating cells were contralaterally injected into the left striatum of the brain (n = 4 in each group). From days 14 to 32, 50 mg/mL GCV was intraperitoneally administered daily and tumor growth in the brain was monitored by bioluminescent imaging of U87-luc cells with the IVIS Imaging System (Figure 3.14 and Figure 3.15). NT2.RA2 migrating-tk cells used together with GCV treatment significantly reduced tumor growth compared with the controls, NT2-tk and NT2.RA2 migrating cells. NT2-tk cells also showed an antitumor effect, though it was not as strong as with the NT2.TA2 migrating-tk cells. At day 32, the U87-luc signal in NT2.TA2 migrating-tk cell–implanted mice was approximately one-sixth of that in the NT2.RA2 migrating group, and approximately one-fourth of that in the NT2-tk group. Compared with the untreated U87 control, it was observed that NT2.RA2 migrating cells themselves might also inhibit tumor growth. Survival of the mice was prolonged by targeted suicide gene therapy (Figure 3.16). At day 32, only one mouse survived in the NT2.RA2 migrating control group, but three mice survived in the NT2.RA2 migrating-tk treatment group. NT2.RA2 migrating cell–mediated bystander effect could significantly inhibit tumor growth and prolong the survival of mice bearing intracranial U87 gliomas.

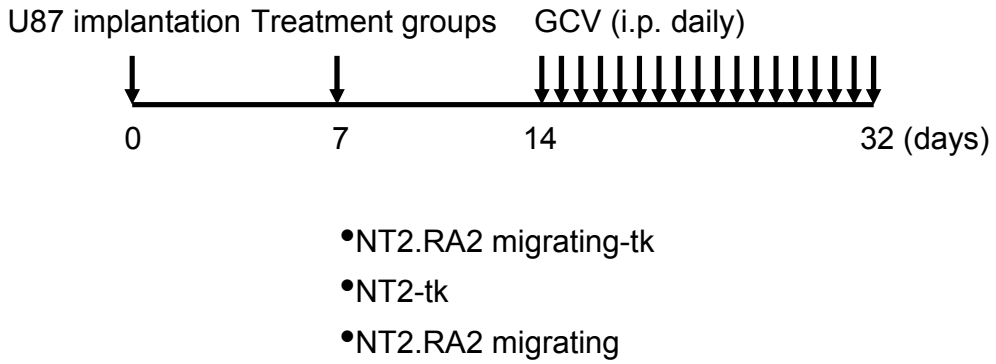

Figure 3.13. Protocol used in the *in vivo* therapeutic effect experiments. 0.5 × 10^6 U87-luc cells were inoculated into the right striatum of the brain. Seven days later, 10^6 NT2.RA2 migrating-tk, NT2-tk, and NT2.RA2 migrating cells were contralaterally injected into the left striatum of the brain (n = 4 in each group). From days 14 to 32, 50 mg/mL GCV was intraperitoneally administered daily.

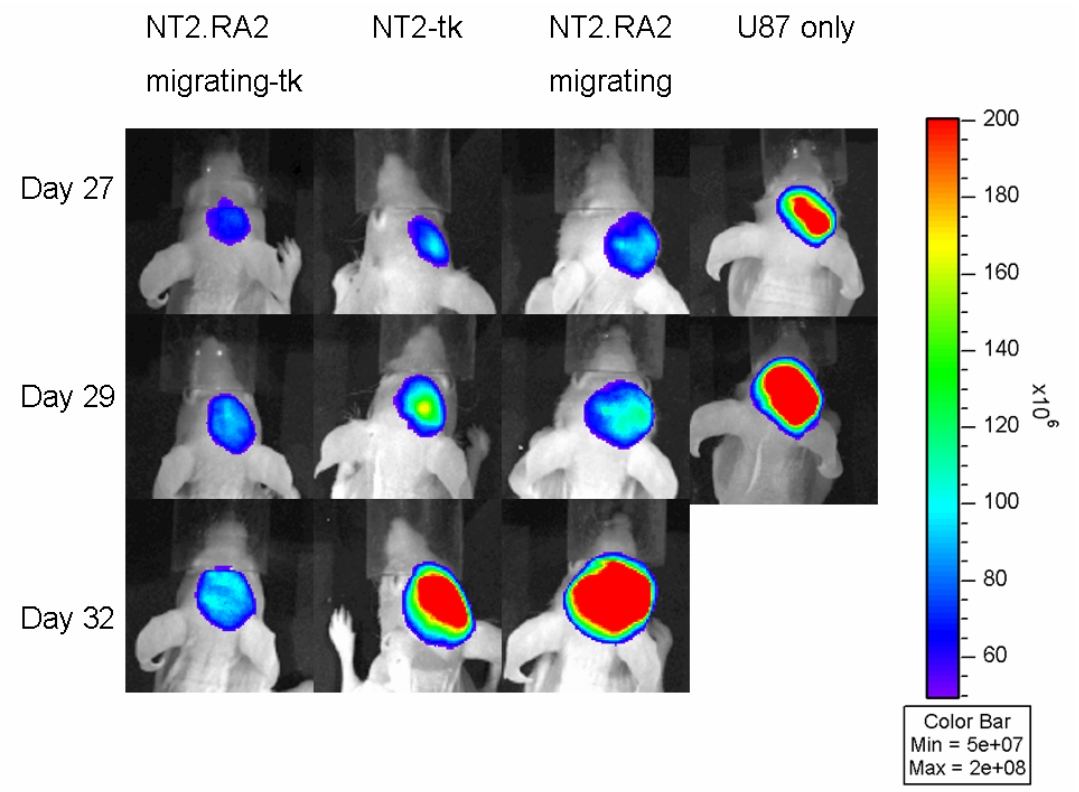

Figure 3.14. *In vivo* therapeutic effect: *in vivo* bioluminescent images of the brain with U87-luc cells inoculation at days 27, 29, and 32 after U87-luc tumor injection. Heat map represents the tumor area and color represents the intensity.

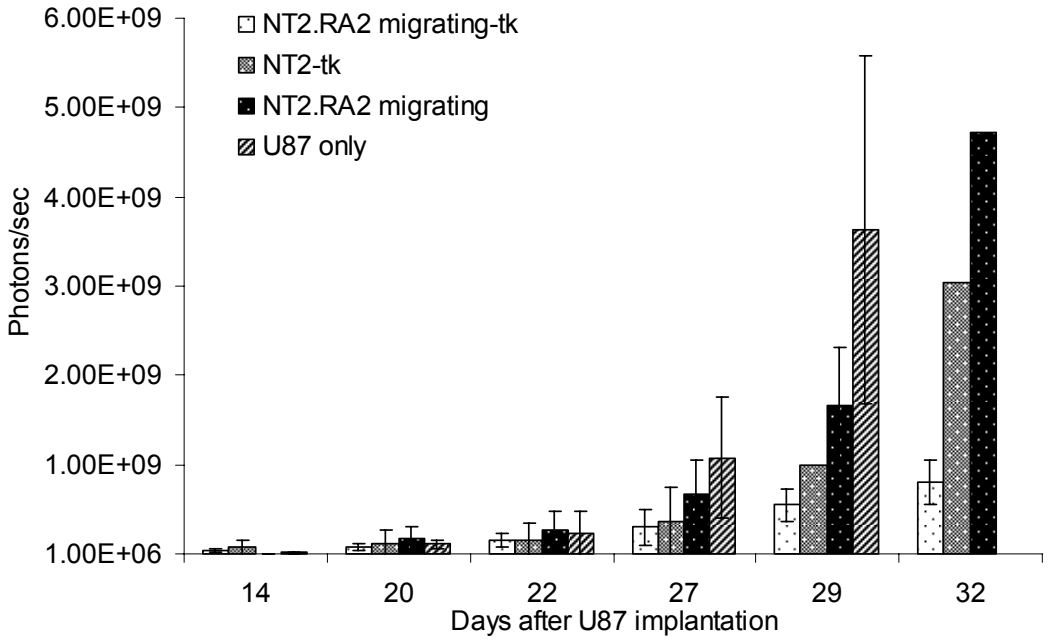

Figure 3.15. *In vivo* antitumor effect: quantification of *in vivo* bioluminescence. Columns: mean bioluminescence intensity (photons/sec); bars: SD.

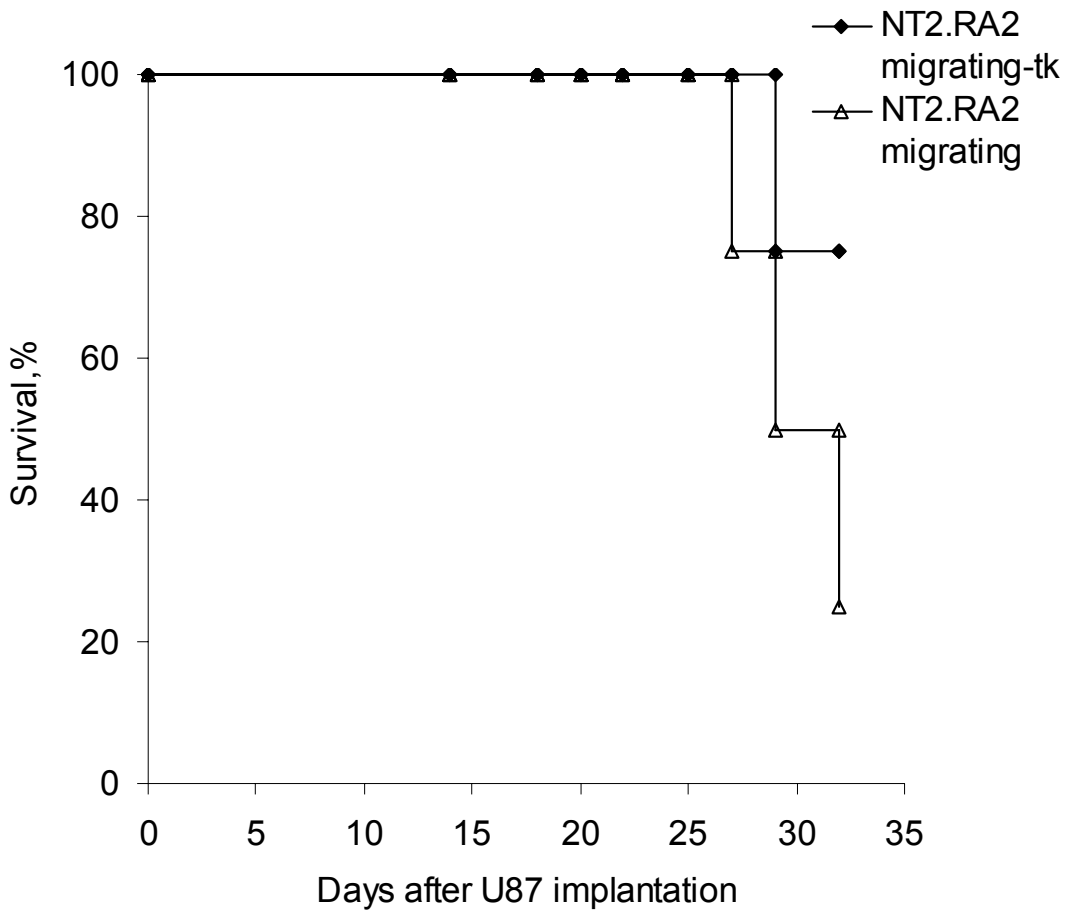

Figure 3.16. Targeted suicide gene therapy mediated by NT2.RA2 migrating-tk cells prolongs survival. Survival curve of eight nude mice in two groups.

3.4 Discussion

A number of reports have demonstrated that HSVtk suicide gene therapy using genetically engineered NSCs might be a promising strategy for glioma therapy (Herrlinger et al, 2000; Li et al, 2007; Li et al, 2005a; Li et al, 2005b; Uhl et al, 2005). However, in these studies, C17.2 cells (immortalized mouse NSC line) or primary fetal-derived rat NSCs were used as gene-delivery vehicles. In this study, we successfully derived glioma tropic human precursor cells from the NPC line, NT2, as an efficient delivery vector for glioma gene therapy. Taking into account their extensive glioma tropism, cell-mediated targeted suicide gene therapy might improve the efficacy of glioma therapy and prolong patient survival.

A population of human glioma tropic precursor cells (NT2.RA2 migrating cells) was derived from RA-treated NT2 cells. These cells offer several advantages when used as gene-delivery vehicles in targeted suicide gene therapy of gliomas. First, like immortalized mouse NSCs and primary rat NSCs, NT2.RA2 migrating cells demonstrate specific glioma cell–directed migration *in vitro* and *in vivo*, and glioma tropism endured during long-term culture. The glioma tropism of NT2.RA2 migrating cells ensures a high concentration of the HSVtk enzyme in the region of the tumor cells. Systemically administered GCV reaches the brain and is activated by the HSVtk expressed by the NT2.RA2 migrating cells in juxtaposition to the tumor cells. NT2.RA2 migrating cells may also be able to deliver antitumor transgenes even after

systemic administration. *In vivo* migration assays showed that, after intravenous administration, NT2.RA2 migrating cells could target subcutaneously implanted U87 tumors as well as intracranially injected U87 gliomas. In the treatment of brain tumors, the BBB is the primary obstacle to successful delivery of molecules to the CNS neoplasm. However, the BBB does not affect the capacity of NSCs to home in on CNS-malignant cells when NSCs are injected into the cerebral ventricles and even into peripheral circulation (Aboody et al, 2000). Therefore, NT2.RA2 migrating cells with HSVtk genes could be implanted in the cavity in the brain during neurosurgery, and the therapeutic effect might be further improved by repeated systemic administrations after surgery. A study illustrated that high glioma tropism is possibly due to the high expression of chemokine and growth factor receptors. The chemokines and growth factors were secreted by tumor cells, attracting the NT2.RA2 migrating cells toward the glioma. In therapeutic models, NT2.RA2 migrating cells were more resistant than NT2 cells to GCV and mediated a stronger bystander effect when expressing HSVtk gene, which could be due to RA-induced cell cycle arrest and slowing of cell proliferation. The doubling time of NT2.RA2 migrating cells is about 3 to 4 days whereas that of NT2 cells is only 24 hours. NT2.RA2 migrating cell–mediated bystander effect kills surrounding tumor cells without HSVtk expression. The phosphorylated GCV is further transferred from a small number of killed tumor cells to bystanders, causing tumor cell death in an even broader region. The bystander effect thus amplifies the therapeutic effect. The use of HSVtk

suicide gene therapy may help to eliminate proliferating NT2.RA2 migrating cells after treatment, thus preventing malignant neoplasm formation (Li et al, 2005a).

NT2.RA2 migrating cells themselves could inhibit tumor growth. Several studies have demonstrated the innate tumor-killing capacity of rodent NSCs (Benedetti et al, 2000; Glass et al, 2005; Staflin et al, 2004). In this study, we further illustrated that the human precursor cells derived from NT2 cells may also have a tumor-inhibitory effect. We observed that NT2.RA2 migrating cells maintained long-term proliferation in common cell-culture conditions, ensuring the preparation of an amount of cells sufficient for transplantation, favorable for *ex vivo* genetic modification and selection.

The potential formation of tumors by NT2.RA2 migrating cells is always a concern. RA treatment suppresses the tumorigenicity of NT2 cells because of RA-induced downregulation of FGF-4 and TGF-α (Newman et al, 2005). Several studies have shown that RA-treated NT2 cells have a remarkably reduced tumorigenicity compared with untreated NT2 cells when subcutaneously injected into athymic mice (Dmitrovsky et al, 1990; Maerz et al, 1998). Three months after RA-treated NT2 cells implantation, no tumor formation was found (Baselga et al, 1993). In addition, the use of HSVtk/GCV suicide gene therapy may help to eliminate proliferating NT2.RA2 migrating

cells after treatment, preventing the formation of malignant neoplasms. Future study is needed to verify the safety of the NT2.RA2 migrating cells *in vivo*.

CHAPTER 4

HUMAN EMBRYONIC STEM CELLS-DERIVED NEURAL STEM CELLS AS DELIVERY VECTORS FOR GLIOMA GENE THERAPY

4.1 Introduction

The use of fetal-derived NSCs is a major limitation in stem cell–based glioma gene therapy. Human NSCs used in experimental models are isolated from the fetal human brain mainly by FACS (Rossi and Cattaneo, 2002), but the process is quite tedious and NSCs are difficult to expand after isolation. Moreover, the fetal source of NSCs raises serious ethical and legal concerns. To overcome the source limitations and the ethical hurdles faced in therapeutic applications, alternative large-scale sources of NSCs must be identified (Martino and Pluchino, 2006).

Human ESC-derived NSCs could provide an unlimited cell source. Self-renewing ESCs are inherently immortal, and their proliferation capacity is preserved during long-term cell culture. The great plasticity of ESCs allows the derivation and isolation of glioma tropic NSCs, which can serve as targeting gene-delivery vehicles in the treatment of patients with malignant glioma. It seems that neuronal fate is most favored by ESCs when there is no other instructive cue (Smukler et al, 2006). Many NSCs and other specific neural cells, such as dopamine and motor neurons, have been differentiated from mouse and human ESCs. A variety of methods have been developed to induce NSCs from human ESCs, but most of methods have used the formation of neurospheres or embryoid bodies (EBs). NSCs could be generated by overgrowth of human ESCs to a higher cell density (Reubinoff et al, 2001). After prolonged culture of human ESCs, without changing the

feeder cells for 3 to 4 weeks, NSC marker-positive cells are isolated mechanically and put into a serum-free medium to form neurospheres. Forming EBs is another way to induce neural differentiation of human ESCs (Carpenter et al, 2001; Zhang et al, 2001). The EBs are subsequently seeded onto an appropriate substrate in a defined medium containing mitogens to further select NSC population. However, the proliferation of NSCs derived by forming neurospheres and EBs is limited in cell culture, and the difficulty of handling cell aggregations limits large-scale preparation. In addition to neurosphere and EB formation, directed differentiation of ESCs to NSCs has been achieved by coculture with mouse PA6 stromal cells (Song et al, 2007). But the exposure to animal cells is always a safety concern in considering about the therapeutic applications. Recently, Smith and colleagues showed that simple plating of mouse and human ESCs in a monolayer culture could successfully achieve NSCs (Conti et al, 2005; Ying et al, 2003). This novel and straightforward method makes the bulk preparation of NSCs from ESCs possible.

We derived NSCs from human ESC lines HES1 and HES3 using a simple adherent monoculture that included bFGF and EGF. The potential applications of these cells in targeted suicide gene therapy of malignant glioma were further explored.

4.2 Materials and methods

4.2.1 Cell culture

The NIH Human Embryonic Stem Cell Registry-listed hES cell lines HES-1 and HES-3 were obtained from ES Cell International (ESI). The hES cells were amplified and maintained according to protocol provided by ES Cell International. The hES cells were cultured on mitotically inactivated mEFs (CF-1, American Type Culture Collection), seeded in gelatin-coated dishes in 80% knockout DMEM (Invitrogen) supplemented with 20% Knockout™ Serum Replacement (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 4 ng/mL bFGF (Invitrogen), 50 U/mL penicillin, and 50 g/mL streptomycin. The hES colonies were subcultured every 7 days by mechanical slicing and replating into fresh feeder layers.

To form embryoid bodies (EBs), hES cells were grown to form large colonies and detached using 0.1 mg/mL dispase (Invitrogen). The hES cell clumps were transferred to a 15 mL conical tube containing 10 mL of a differentiation medium consisting of 80% knockout DMEM, 20% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, and 0.1 mM nonessential amino acids and allowed to settle to the bottom. The supernatant was removed. The cell clumps were resuspended in the differentiation medium and transferred to a Petri dish. The cells were fed every day by replacing half the medium with fresh differentiation medium and were cultured for 2 weeks.

U87 MG (ATCC) and H4 (ATCC) glioma cell lines were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 2 mM glutamine, and 0.1 mM nonessential amino acids. 293FT cell line was purchased from Invitrogen, maintained in a basal medium containing 500 μg/mL geneticin (GIBCO).

4.2.2 Neural differentiation of hES cells

For monoculture differentiation of HES1 and HES3 cells, hES cell colonies were detached from the organ dish 7 days after plating by mechanical cutting. hES cells were then dissociated using trypsin and plated onto a 0.1% gelatin-coated six-well cell-culture plate at a density of 10^6/well and cultured in NSC medium. NSC medium was a 1:1 mixture of DMEM/F12 (GIBCO) supplemented by 2% B27 (GIBCO), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 20 ng/mL EGF (Sigma), and 20 ng/mL bFGF (Invitrogen). Half the cell-culture medium was changed every 2 days. At 7 days of differentiation, cells reached 90% confluence and were split at 1:2. After 1 month of expansion, NSC lines NSC1 and NSC3 were derived from HES1 and HES3. Cells were digested using TrypLE Express Dissociation Enzyme (GIBCO) for NSC1 and NSC3 cell passage and subcultured at ratio of 1:2 twice weekly.

To induce neural differentiation of NSCs, cells were transferred to a low–cell binding six-well plate (NUNC) in NSC medium. After culture of 1 to 3 weeks, round neural spheres were formed. The spheres were then plated into poly-D-lysine (Sigma)- and laminin (Sigma)-coated dish. Neuronal and glial differentiation was induced by the withdrawal of grow factors EGF and bFGF from the culture medium.

4.2.3 Immunocytochemistry and FACS analysis

Cells were washed with PBS and fixed at room temperature with 4% paraformaldehyde for 10 minutes and permeated with 0.1% triton for 10 minutes. The cells were then blocked with 5% normal goat serum for 1 hour and incubated overnight at 4°C with antinestin antibody (1:200; Chemicon), anti-NCAM antibody (1:200; Santa Cruz Biotechnology), anti-A2B5 antibody (1:200; Chemicon), anti-β tubulin III monoclonal antibody (1:200; Promega), anti-GFAP (1:200; Sigma), and anti-O4 antibody (1:100; Chemicon). Goat antimouse IgG-FITC antibody (Sigma) and Goat antirabbit IgG-FITC antibody (Sigma) were then applied for 60 minutes to visualize the antigens. In immunocytochemistry, 4', 6-diamidino-2-phenylindole (DAPI, 2 nM, Sigma) was applied before observation. Immunofluorescence was visualized and captured using an Olympus image analysis system. In FACS analysis, the cells were analyzed with a FACSCalibur™ flow cytometer (BD) for the percentage of fluorescence-positive cells.

4.2.4 Reverse transcription- PCR

Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). One microliter of cDNA reaction mix was subjected to PCR amplification using PCR SuperMix (Invitrogen), as recommended by the manual. Reactions were subjected to 30 PCR cycles after denaturation at 94°C for 4 minutes, as follows: 94°C for 30 seconds; 55°C for 30 seconds; and 72°C for 60 seconds or 2 minutes. An extension step of 72°C for 5 minutes was included. All products were electrophoresised on a 2% agarose gel. The forward and reverse primers and sizes of RT-PCR productions were as follows: Oct-4, 169 bp, 5'-CTTGCTGCAGAAGTGGGTGGAGGAA-3' (forward) and 5'-CTGCAGT GTGGGTTTCGGGCA-3' (reverse); Nanog, 426 bp, 5'-GCGCGGTCTTGGCT CACTGC-3' (forward) and 5'-GCCTCCCAATCCCAAACAATACGA-3' (reverse); Brachyury T, 284 bp, 5'-CAACCACCGCTGGAAGTAC-3' (forward) and 5'-CCGCTATGAACTGGGTCTC-3' (reverse); AFP, 675 bp, 5'-AGAACCT GTCACAAGCTGTG-3' (forward) and 5'-GACAGCAAGCTGAGGATGTC-3' (reverse); β-actin, 513 bp, 5'-GCCCAGAGCAAGAGAGGCAT-3' (forward) and 5'-GGCCATCTCTTGCTCGAAGT-3' (reverse); HSVtk, 593 bp, 5'-CAATCG CGAACATCTACACCACA-3' (forward) and 5'-CCGAAACAGGGTAAA TAACGTGTC-3' (reverse).

4.2.5 Lentivirus preparation and genetic engineering

HSVtk gene was cloned from expression vector pORF-HSVtk (InvivoGen) by PCR using primers 5'-CACCATGGCCTCGTACCCCGGCCATC-3' and 5'-TCAGTTAGCCTCCCCCATCTCCCGG-3' into pLenti6/v5-TOPO vector (Invitrogen) followed by sequencing to confirm construction. HSVtk lentiviruses were produced using the ViraPower Lentiviral Directional TOPO Expression Kit (Invitrogen). In brief, HSVtk lentiviruses were packaged in 293FT cells by cotransfection of the expression vector pLenti6/v5-HSVtk and the packaging plasmids (pLP1, pLP2 and pLP/VSVG). Lentiviral supernatants were harvested 48 hours after transfection and filtered through a 0.45 μM membrane. Virus suspension was centrifuged at 50,000 rpm and 4°C for 2 hours to concentrate the lentivirus. The virus particles were resuspended with DMEM, stored at –80°C, or immediately used in transduction. In genetic engineering of NSC1 cells, cells were transduced overnight by HSVtk lentivirus in 6 μg/mL polybrene (Invitrogen), followed by blasticidin selection at a concentration of 5 μg/mL for 2 weeks.

4.2.6 *In vitro* migration assay

The directed migration ability of stem cells was determined by a modified Boyden chamber assay, with the BD Falcon HTS FluoroBlok 96-Multiwell Insert System (8 μm pore size). One day before the migration assays, glioma cells were seeded at a density of 6.4×10^4/well in 96-well companion plates (BD Falcon) and the medium was replaced with 200 μL Opti-MEM (Invitrogen).

Stem cells were labeled with Calcein-AM (molecular probes). During labeling, cells were incubated with 5 μg/mL Calcein-AM in culture medium for 10 minutes and then washed three times with culture medium, each time for 10 minutes. Labeled cells were starved overnight in Opti-MEM. The next day, 96-multiwell cell-culture inserts were put into the 96-well companion plates. The labeled stem cells were suspended with Opti-MEM and seeded into multiple inserts at 2.5×10^4 /insert. The plates and inserts were incubated together for 24 hours at 37°C in 5% CO_2. The fluorescence from the top side (corresponding to nonmigrating cells) and the bottom side (corresponding to migrating cells) of the plates was measured using a microplate reader (GENios Pro, Tecan). Fluorescence background was subtracted during calculation. Values were calculated as the percentage of the bottom reading in the total reading. All experiments were conducted in quadruplicate and values were expressed as mean ± SD. Statistical analyses were made using Student's *t* test.

4.2.7 *In vitro* bystander effect

To test the cytotoxicity of GCV, 10^3 NSC1 cells, NSC1-tk cells, U87 cells, a coculture at ratio of 1:1 of NSC1-tk cells and U87 cells was seeded in a 96-well cell-culture plate. Cells were cultured in a medium containing 0.1 μg/mL, 1 μg/mL, and 10 μg/mL GCV, and the conditional medium was changed every 2 days. The cell numbers were determined by CellTiter 96 AQueous Assay (MTS, Promega) on day 10. Cell viability was calculated as the percent

absorbance of cells cultured without GCV. Values from six wells were expressed as mean ± SD and statistical analyses were made using Student's *t* test.

4.3 Results

4.3.1 Self-renewing neural stem cells are derived from human embryonic stem cells by adherent monoculture

Human ESCs, HES1 and HES3, were converted to NSCs, NSC1 and NSC3, in a serum-free adherent monoculture. To induce neural differentiation, ESCs were dissociated and plated onto the 0.1% gelatin-coated cell-culture plate at a high density, in serum-free medium with EGF and bFGF. Approximately 50% cells died after being transferred to serum-free medium due to failure to attach. However, after medium changing, abundant viable and proliferative cells remained inside the cell-culture plate. At as early as 7 days of differentiation, a high percentage of bipolar cells similar to NSCs was observed. After 1 month of expansion, homogenous NSC lines were achieved (Figure 4.1). NSC1 and NSC3 were routinely split into two twice per week. TrypLE Express Dissociation Enzyme, which does not require the serum deactivation and is less toxic than trypsin, was used in the digestion and detachment of NSC cells. The NSC lines were continuously cultured for at least 6 months and maintained proliferation (data not shown). Furthermore, NSC1 and NSC3 could be cryopreserved in an NSC medium plus 10% DMSO and were recoverable from liquid nitrogen (data not shown). Long-term proliferating NSC lines were successfully derived from human ESCs in a defined adherent culture condition with growth factors bFGF and EGF.

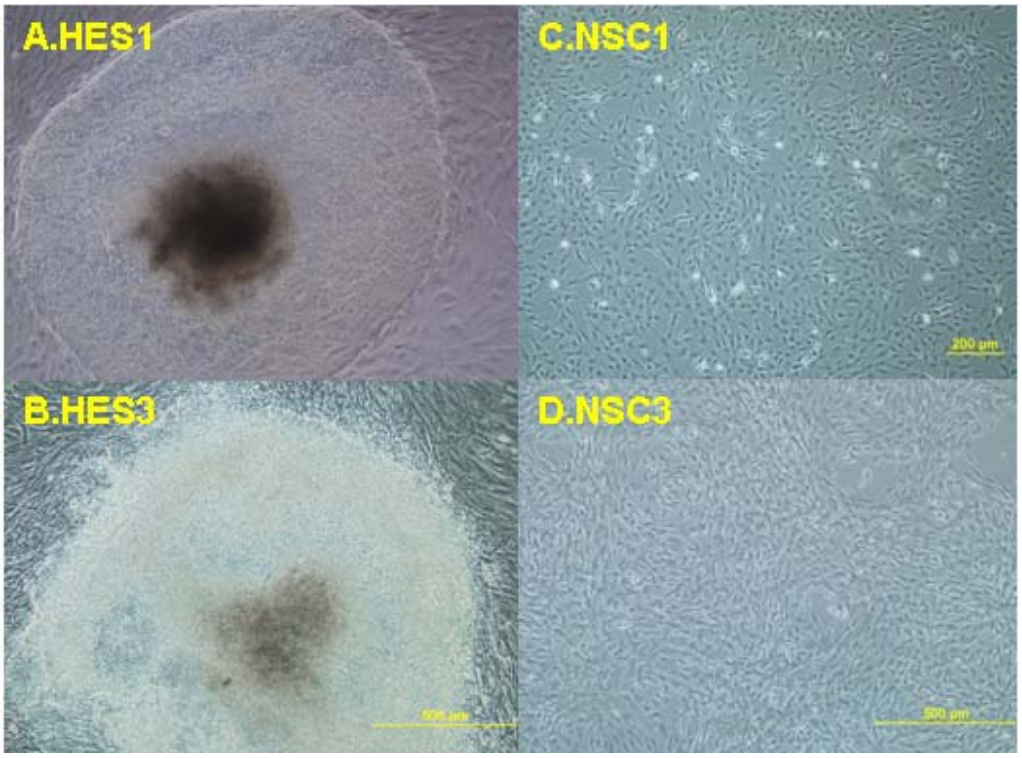

Figure 4.1. Self-renewing NSC lines NSC1 and NSC3 are derived from hES cells. HES1 (A) and HES3 (B) ESCs are cultured on the feeder cells. NSC1 (C) and NSC3 (D) NSCs are cultured as monolayers.

4.3.2 “Stemness” of human embryonic stem cell-derived neural stem cells

To characterize NSCs derived from ESCs, we examined the expression of the NSC markers and the neural multipotency of these cells. Immunocytochemistry analysis showed that both NSC1 and NSC3 cells were NSC marker positive (Figures 4.2 and 4.3). Nestin is an early-stage NSC marker, NCAM is a late-stage neural lineage stem cell marker, and A2B5 is a glia lineage marker. Nestin, NCAM, and A2B5 were all expressed in NSC1 and NSC3 cells. This was further confirmed by FACS analysis (Figure 4.4). Ninety-eight percent of NSC1 cells were nestin positive, 90% were NCAM positive, and 97% were A2B5 positive. Through RT-PCR analysis (Figure 4.5), NSC1 and NSC3 cells were found to lack pluripotent stem cell markers Oct-4 and Nanog. Unlike EBs, the transcripts of mesoderm mark, Brachyury T, and endoderm marker, AFP, were not detected in NSC1 and NSC3 cells.

When transferred to cell suspension culture, NSC1 cells formed neural spheres, with the presence of bFGF and EGF in culture medium (Figure 4.6). After sequential withdrawal of bFGF and EGF, they generated mixed populations of β-Ш tubulin–positive neuron cells and GFAP–positive glia cells (Figures 4.6 and 4.7). These results support the fact that NSC1 and NSC3 are NSCs with high purity and have the potential to differentiate to neurons and glias.

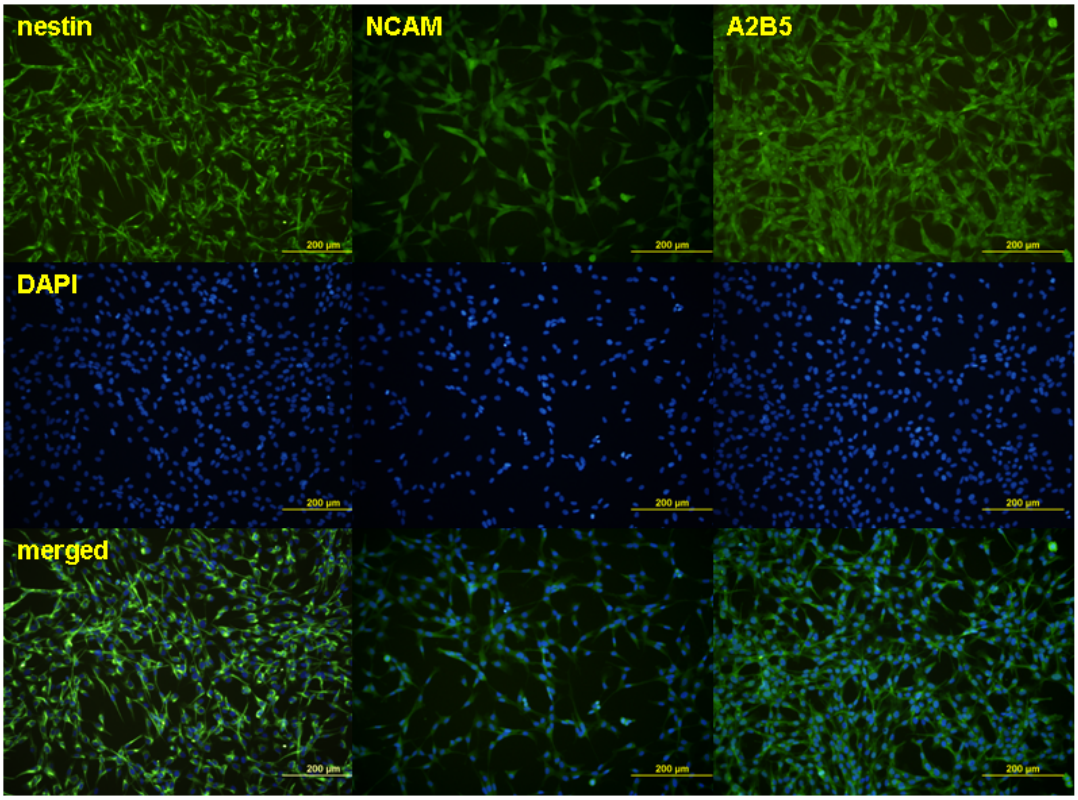

Figure 4.2. Nestin, NCAM, and A2B5 expression in NSC1 cells. Fluorescence photographs show the immunostaining using antibodies against nestin, NCAM, and A2B5 (green). DAPI was used to counterstain nuclei (blue).

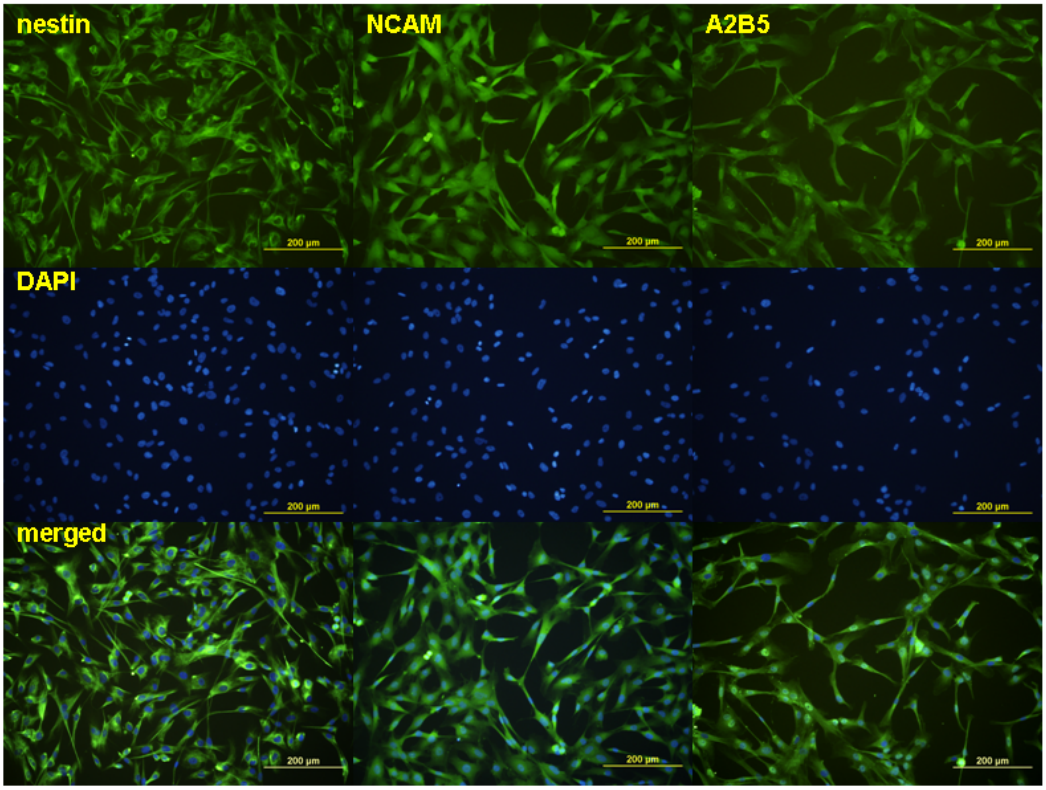

Figure 4.3. Nestin, NCAM, and A2B5 expression in NSC3 cells. Fluorescence photographs show the immunostaining using antibodies against nestin, NCAM, and A2B5 (green). DAPI was used to counterstain nuclei (blue).

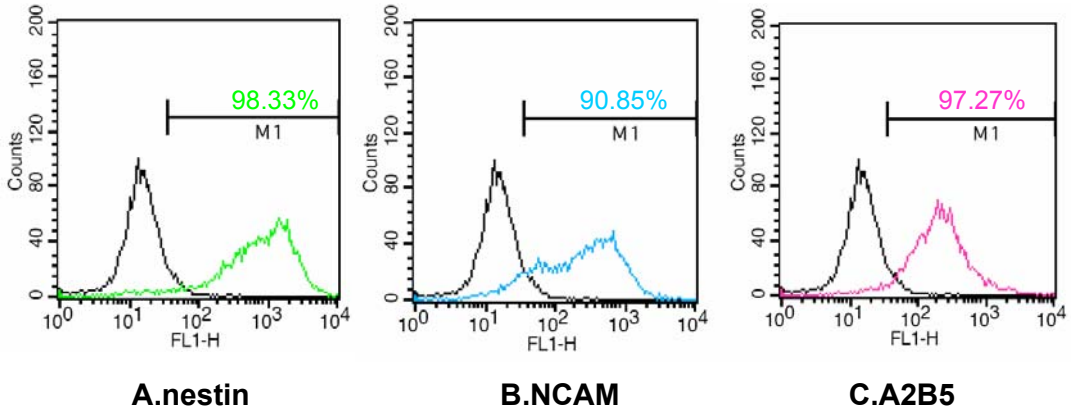

Figure 4.4. FACS analysis of neural stem marker expression on NSC1 cells. Cells incubated only with FITC-conjugated secondary antibodies were used as control. (A) nestin; (B) NCAM; (C) A2B5.

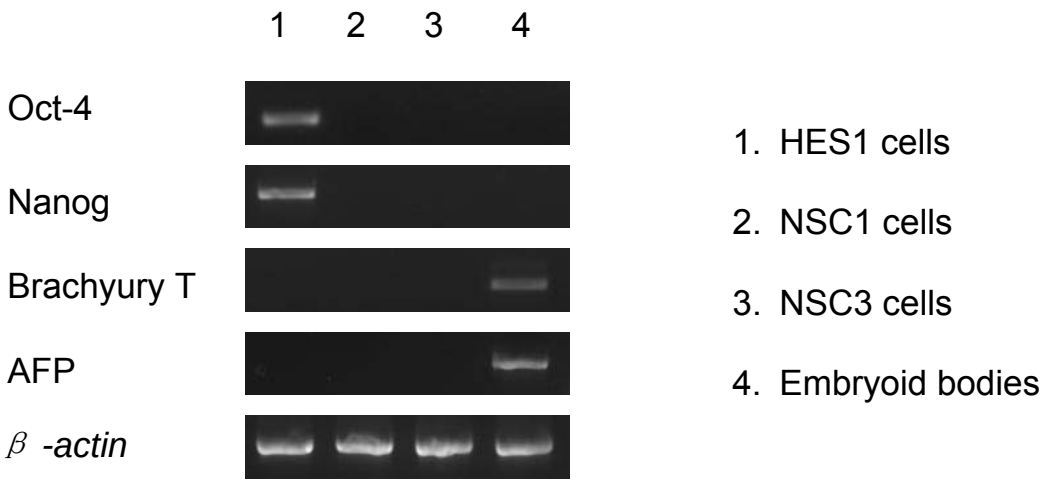

Figure 4.5. The analysis of stem cell markers using RT-PCR. The expressing levels of Oct-4, Nanog, brachyury T, and AFP in HES1, NSC1, NSC3, and EBs were confirmed and visualized on 2% agarose gel.

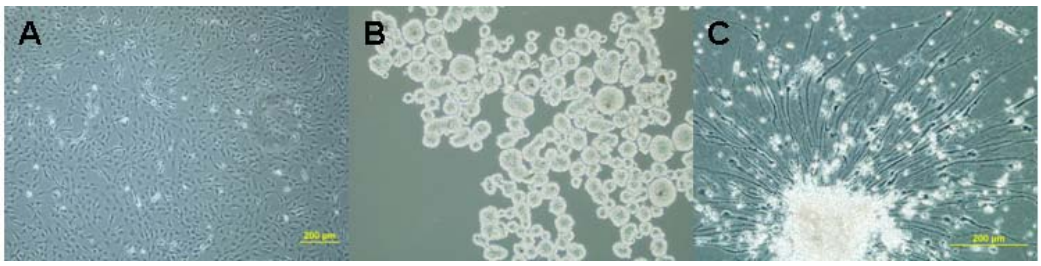

Figure 4.6. Neural differentiation of ESC-derived NSCs. NSC1 cells were cultured in monoculture (A). In suspension culture, NSC1 cells formed neural spheres (B). After withdrawal of bFGF and EGF, neural spheres further differentiated to a mixed population of neurons and glias (C).

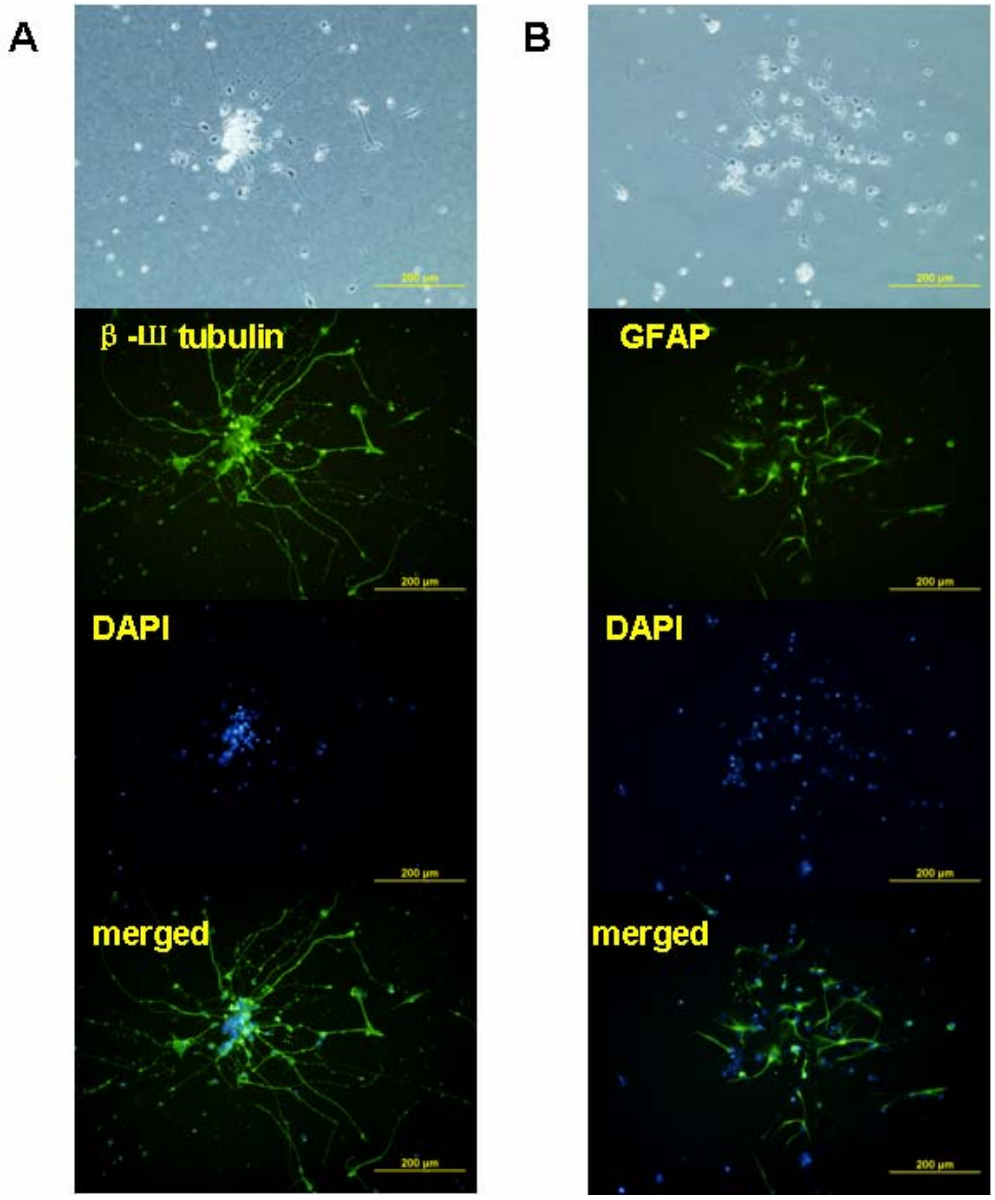

Figure 4.7. ESC-derived NSCs give rise to neurons and glias. Fluorescence photographs show the immunostaining using antibodies against β-III tubulin and GFAP (green). DAPI was used to counterstain nuclei (blue).

4.3.3 *In vitro* glioma tropism evaluation of human embryonic stem cell-derived neural stem cells

The extensive glioma tropic behavior of primary NSCs and NSC lines has been well documented. We used the modified Boyden chamber migration assay to test the capacity of human ESCs derived from NSCs to migrate toward gliomas. Mouse NSC C17.2 was used as a positive control and mouse fibroblast cell 3T3 was used as a negative control. We examined the migration of NSC1, NSC3, C17.2, and 3T3 toward plain medium (MEM), human kidney cell line (293FT), and human glioma cell lines (U87 and H4). Results were obtained using a fluorescence microscope (Figure 4.8) and fluorescence microplate reader (Figure 4.9). The results of *in vitro* migration assays showed that all NSCs (NSC1, NSC3, and C17.2) had a large number of cells migrating toward the glioma cells (U87 and H4), but not to MEM and 293FT. Far fewer 3T3 cells migrated to glioma cells, and there was no difference between the migration toward glioma and nonglioma cells. When comparing the glioma-directed migration of three NSC lines, NSC3 was at a level similar to that of C17.2, whereas NSC1 showed the greatest glioma-specific migration. The glioma tropism of ESC-derived NSCs makes them well suited as delivery vectors for glioma gene therapy.

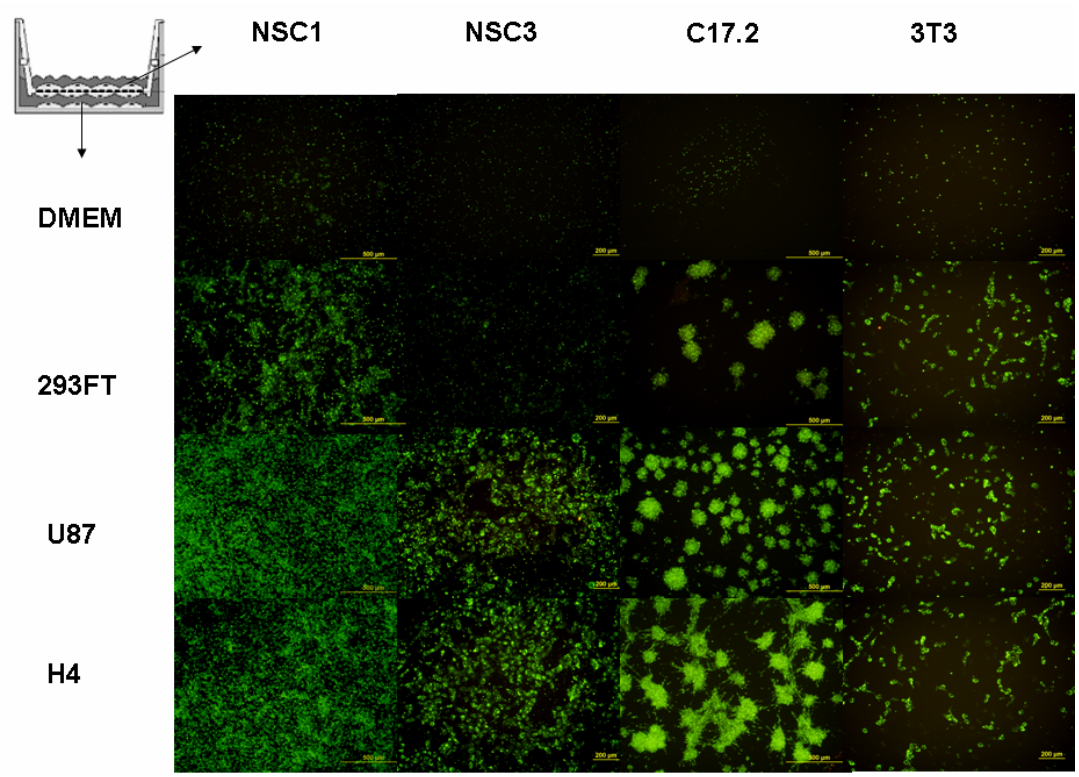

Figure 4.8. *In vitro* migration of ESC-derived NSCs toward glioma cells. Fluorescence photographs show the Calcein-AM–labeled transmigrated NSC1, NSC3, C17.2, and 3T3 toward MEM, 293FT, U87, and H4 from the bottom side in Boyden chamber assays.

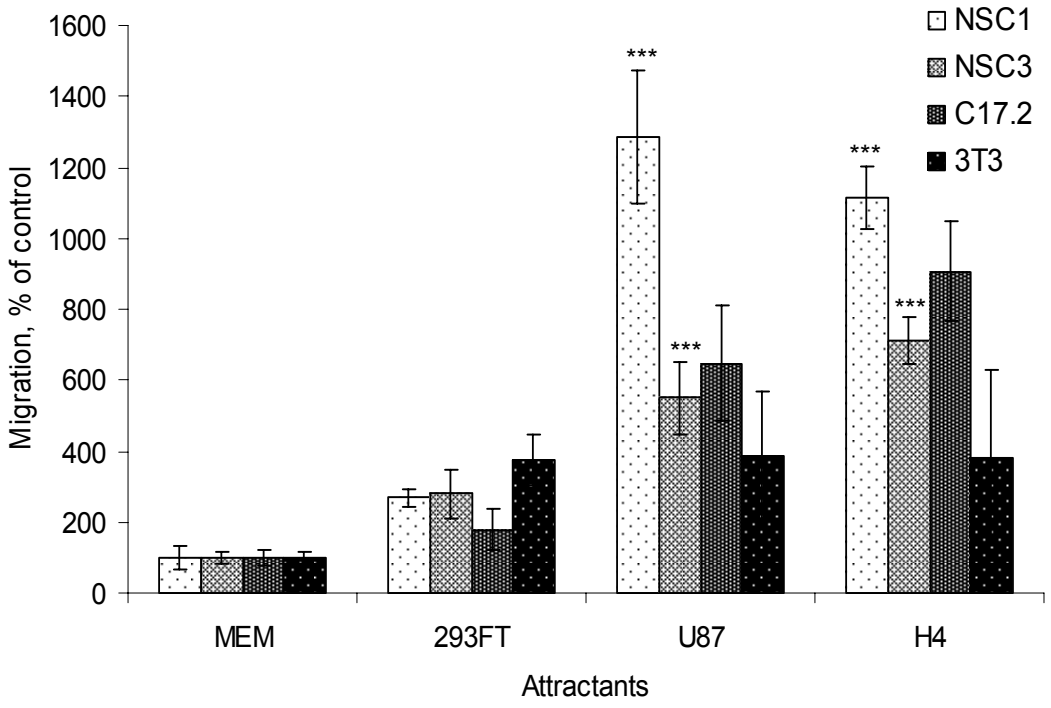

Figure 4.9. Glioma-specific tropic behavior of ESC-derived NSCs. *In vitro* migration of NSC1, NSC3, C17.2, and 3T3 to MEM, 293FT, U87, and H4 was measured by a Tecan microplate reader. Columns: percentage of MEM control; bars: SD. Statistical analysis of MEM control calculated using Student's *t* test. ****P* <0.001.

4.3.4 Human embryonic stem cell-derived neural stem cells as vectors for glioma gene therapy

Lentivirus-transduced NSC1 cells expressing HSVtk transgene, NSC1-tk, were prepared. Expression of HSVtk transcript was confirmed by reverse transcription-PCR (Figure 4.10). The HSVtk transcript was found to be expressed in NSC1-tk cells, but not in the parental NSC1 cells.

The therapeutic potential of NSC1-tk was evaluated in direct coculture experiments. To test their sensitivity to GCV, NSC1, U87, NSC1-tk, and U87+NSC1-tk (1:1) cells were cultured for 10 days in the presence of various concentrations of GCV (0.1 μg/mL-10 μg/mL). The cell viability observed under the microscope is shown in Figure 4.11 and results of the MTS assays are shown in Figure 4.12. Cells cultured in a medium without GCV were used as controls. No toxicity of GCV has been observed in U87 and NSC1 cells. NSC1-tk cells together with GCV significantly inhibited U87 cell growth *in vitro*. At a GCV concentration of 10 μg/mL, nearly all tumor cells were eradicated. However, the NSC1-tk cells by themselves could withstand the effect of phosphorylated GCV. This chemoresistance of ESC-derived NSCs also contributes to the antitumor effect. These results indicated that NSC1-tk cells can convert sufficient amounts of GCV to effectively kill U87 cells *in vitro*.

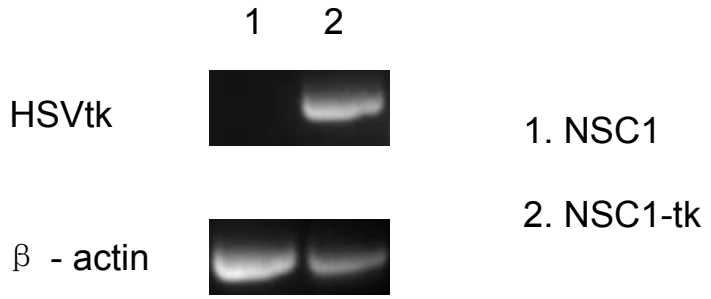

Figure 4.10. The analysis of HSVtk expression using reverse transcription-PCR.

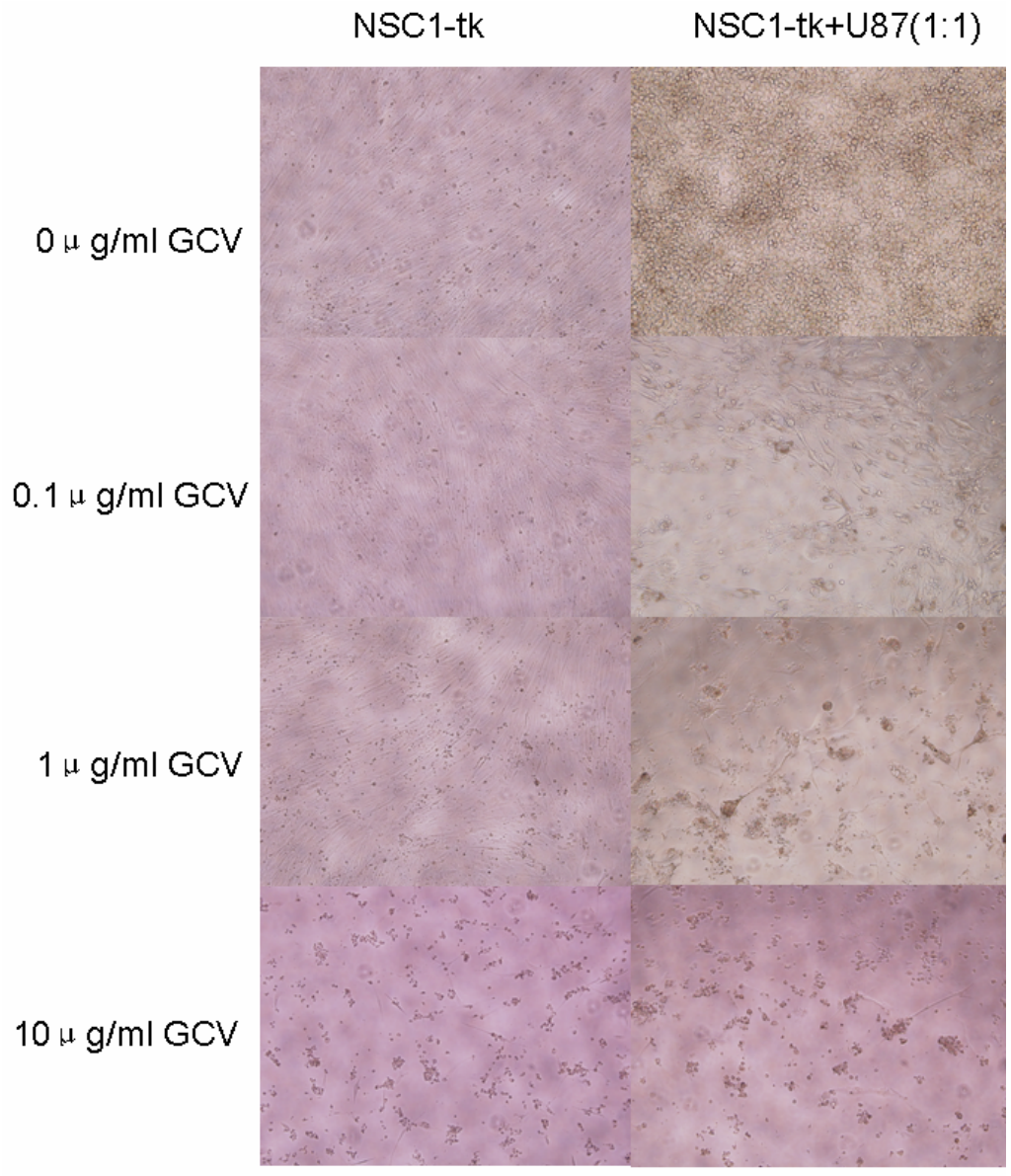

Figure 4.11. Phase contrast images showing the cytotoxicity of GCV in NSC1-tk cells and NSC1-tk cocultured with U87.

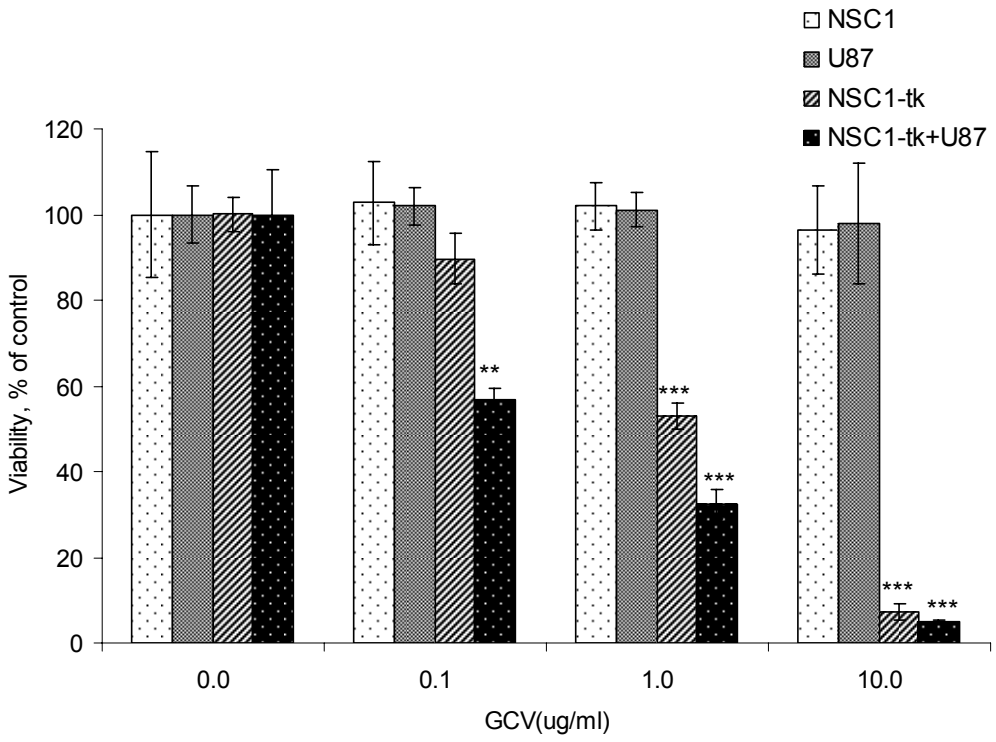

Figure 4.12. *In vitro* therapeutic efficacy of NSC1-tk cells in the coculture system (6 repeats). Columns: mean cell viability (percentage of control cells cultured without GCV); bars: SD. Statistical comparisons to controls calculated using two-factor ANOVA and Student's *t* test. ***P* <0.01; ****P* <0.001.

4.4 Discussion

NSCs have a great capacity to home in on brain tumors and show a specific affinity for invading glioma cells. NSCs may be ideal gene-delivery vehicles for gene therapy of glioma because of their inherent tumor-tropic, tumor-killing and CNS damage–repairing properties. Genetically engineered NSCs that express a therapeutic gene can inhibit the growth of gliomas, facilitate elimination of tumor cells, and repair damaged brain tissue directly. However, the clinical relevance of using cells of fetal origin is limited by significant ethical issues.

Primary adult NSCs may be a viable choice when considering the similarity between primary and endogenous NSCs, as well as the encouraging results in primary rodent NSC-mediated targeting glioma gene therapy (Ehtesham et al, 2002a; Ehtesham et al, 2002b; Li et al, 2007; Li et al, 2005; Shah et al, 2005). However, unlike isolating haematopoietic stem cells (HSCs) from bone marrow, the procedure to derive primary NSCs from the adult brain is extremely invasive, and offers only a low yield. In cell culture, human adult NSCs express low levels of telomerase and stop proliferation after serial passaging (Ostenfeld et al, 2000), so optimized protocols to expand adult NSCs *in vitro* will be required. Because the behavior of NSCs derived from diverse sources and maintained under different culture conditions may vary *in vivo*, researchers should establish standards for the isolation, expansion, and characterization of adult NSCs. Immortalized NSC lines (eg, v-*myc*

immortalized mouse fetal NSC line C17.2 and human fetal NSC line HB1.F3) have shown an outstanding migratory capacity and antitumor effect in a variety of experimental brain tumor models (Aboody et al, 2000; Aboody et al, 2008; Aboody et al, 2006; Danks et al, 2007; Dickson et al, 2007; Herrlinger et al, 2000; Kim et al, 2005; Kim et al, 2006; Shimato et al, 2007). Compared with primary NSCs, immortalized NSC lines are well characterized and can be propagated indefinitely with defined properties on a large scale. The quality of cells used in implantation can be easily controlled by comprehensive analysis of cell lines. However, the utility of oncogenes during immortalization and the potential tumorigenicity of cell line *in vivo* raises safety concerns. The transplantation of allogeneic NSC lines may cause immune rejection, although NSCs have been reported to have low immunogenic potential in their undifferentiated state (Hori et al, 2007).

Human ESCs derived from NSCs might provide an unlimited source of cells for therapeutic applications and offer several advantages over other types of stem cells. Self-renewing ESCs are inherently immortal and their proliferation capacity is preserved throughout long-term cell culture. So far, 21 independent human ESC lines, characterized by NIH using universally accepted criteria, are commercially available worldwide, suggesting that all labs may start from the same cell populations. The great plasticity of ESCs allows the derivation and isolation of glioma tropic NSCs, which can serve as targeting gene-delivery vehicles in the treatment of patients with malignant

glioma. Several strategies have been developed to differentiate NSCs from human ESCs, and the ESC-derived NSCs have demonstrated extensive migratory ability and have differentiated into neurons, astrocytes, and oligodendrocytes in normal and lesioned rat brains (Tabar et al, 2005).

In this study, we demonstrated, for the first time, that human ESCs could provide a potentially unlimited source for glioma gene therapy. We successfully derived long-term proliferating NSCs from HES1 and HES3 human ESCs using a novel monolayer culture condition without cell-aggregation formation. Unlike in previously reported neural sphere differentiation protocols, which normally take several months, only 1 month was required in this study to produce pure NSCs from human ESCs using this monolayer differentiation method. Ninety-seven percent of NSC1 cells were NSC marker nestin positive, indicating a high purity in this population. In addition, neuron precursor marker N-CAM and glia precursor marker A2B5 were positive in NSC1 and NSC3, meaning that NSC1 and NSC3 include both subpopulations of NSCs, neuron precursors and glia precursors. NSC1 and NSC3 formed neural spheres in suspension culture, which is a universally accepted standard for NSCs. When put into the differentiation medium, NSC1 progressed into neurons and glias. The special features of NSC1 and NSC3 make them quite suitable for clinical applications. NSC1 and NSC3 proliferated for at least 6 months in cell-culture conditions while their neuronal

multipotency was preserved. In addition, the cells are expanded in a defined medium, making it easier to control quality and scale up the culture.

The strong glioma-specific tropism of NSC1 cells makes targeting gene delivery possible. *In vitro* migration assays showed that NSC1 and NSC3 cells migrated specifically to glioma cell lines, with a tropism similar to that of C17.2, the best-studied NSC cell line. When carrying the suicide gene HSVtk, NHES1 cells demonstrated strong antitumor effects *in vitro* because of their resistance to phospha-GCV. It has been reported that NSCs are more resistant to toxins because of the high level of expression of the ATP binding cassette transporter, which makes NSCs more efficient than other cellular vectors in suicide gene therapy (Dean et al, 2005).

When stem cells are used in therapeutic application, tumorigenesis is always a concern. Even a single undifferentiated ESC possesses the ability to form teratoma after transplantation. Here, we have shown that pluripotent stem cell markers were not expressed in NSC1 and NSC3 cells. The safety and efficiency of this ESC-derived vector should be further investigated in large-scale preclinical studies. Immunogenicity might be another problem posed by allogeneic ESC-derived NSCs, but the breakthrough in derivation of human iPS cells may overcome this limitation. At the end of 2007, two groups reported successful reprogramming of human somatic cells to iPS cell lines with defined transcription factors, using either the same set of transcription

factors (Takahashi et al, 2007) or another set, Oct4, Sox2, Nanog, and Lin28 (Yu et al, 2007). This cellular reprogramming technique allows the derivation of patient-specific pluripotent stem cells from their own somatic cells; thus, no ethical issues arise. NSCs derived from iPS cells might provide an autologous cell source for glioma gene therapy, which could eliminate the immune rejection induced by other types of stem cells.

Chapter 5

Conclusions

This work aimed to discover new regulators able to enhance cell migration toward gliomas and to develop alternative, large-scale cell sources of NSCs for glioma gene therapy. In this thesis, we presented three approaches for utilizing and optimizing the NSC vector in gene therapy of glioma.

In Chapter 2, we described the identification and characterization of a novel cell motility modulator, TMEM18. TMEM18 was first identified in a screening searching for membrane-spanning proteins. Based on our results, the function of TMEM18 would probably be linked to cell mobility. TMEM18 could be used as a specific enhancer for glioma-directed migration of NSCs. However, knockdown of endogenous TMEM18 expression with RNA interference has enormous inhibitory effects on the overall movement of NSCs. Likewise, along with the increase of TMEM18 expression from an undetectable to an easily detectable level when human ESCs differentiated into NPCs, these NSC/NPCs displayed an increased capacity for cell migration. These findings indicated a crucial role of the basal, physiological level expression of TMEM18 for cell movement, which is consistent with the highly conserved and ubiquitously expressed pattern of TMEM18. Most interestingly, TMEM18-overexpressing cells respond strongly to glioma cell–secreted cues, as observed *in vitro* transwell assays and in an *in vivo* migration experiment. We concluded that TMEM18 overexpression increases the sensitivity of NSCs to appropriate signals that stimulate cell migration. Without appropriate cues, TMEM18 overexpression will have undetectable effects on the movement of

NSCs. Our preliminary data on the upregulation of CXCR4 in TMEM18-overexpressing cells and on the inhibition of cell migration by antibodies against CXCR4 suggested an enhanced effect of the SDF-1/CXCR4 axis by TMEM18. Further research is necessary to define the basic mechanisms underlying the effects of endogenous TMEM18 on general cell migration and the effects of overexpressed TMEM18 on enhancing cell response to migration-stimulating signals. An adequate understanding of these mechanisms could have important implications for effective cellular delivery of therapeutic agents in brain tumor therapy.

In Chapter 3, a population of human glioma tropic precursor cells was derived from RA-treated NT2 (NT2.RA2) cells. RA treatment induced neuron differentiation of NT2 cells and improved migration capacity toward U87 cells. RA treated cells showed the greatest glioma tropism after 2 weeks treatment. Their migration toward glioma cells was further improved by migration screening. The enhanced migration capacity of NT2.RA2 migrating cells is glioma specific and preserved during long-term culture. At the molecular level, this high glioma tropism is possibly because of the high expression of chemokine and growth factor receptors. The chemokines and growth factors were secreted by tumor cells, attracting the NT2.RA2 migrating cells toward gliomas. In nude mice with subcutaneously implanted U87 gliomas and intracranially injected U87 gliomas, NT2.RA2 migrating cells were able to target tumor cells after tail-vein injection. In addition to enhanced glioma tropic

behavior, NT2.RA2 migrating cells were more resistant to GCV and, when expressing HSVtk gene, mediated a stronger bystander effect than NT2 cells. This may be because of RA-induced cell cycle arrest and slowing of cell proliferation. In a therapeutic model, NT2.RA2 migrating cells delivered the HSVtk gene to the tumor site and mediated a strong antitumor effect. In addition, the survival of nude mice with brain tumors was prolonged after 2 weeks of GCV administration. We had successfully derived glioma tropic precursor cells from the NPC line NT2 and used them as efficient delivery vectors for gene therapy of glioma. This cell-mediated targeted suicide gene therapy may improve the efficacy of glioma therapy and prolong patient survival. However, considering the carcinomal origin of these cells, the safety of the NT2.RA2 migrating cells as a therapy should be further characterized.

In Chapter 4, we demonstrated that human ESCs could provide a potentially unlimited source for glioma gene therapy. We successfully derived long-term proliferating NSCs from HES1 and HES3 human ESC lines using a novel monolayer culture condition. Unlike in previously reported neural sphere differentiation protocols, which normally take several months, only 1 month was required in this study to produce pure NSCs from human ESCs using this monolayer differentiation method. Ninety-seven percent of NSC1 and NSC3 cells were NSC marker nestin positive, indicating a high purity in this population. In addition, neuron precursor marker N-CAM and glia precursor marker A2B5 were also positive in NSC1 and NSC3, meaning that NSC1 and

NSC3 include both subpopulations of NSCs, neuron precursors and glia precursors. NSC1 and NSC3 formed neural spheres in suspension culture, which is a universally accepted standard for NSCs. When put into the differentiation medium, NSC1 progressed into neurons and glias. The special features of NSC1 and NSC3 make them quite suitable for clinical applications. NSC1 and NSC3 proliferated for at least 6 months in cell-culture conditions, and their neuronal multipotency was preserved. In addition, the cells are expanded in a defined medium, making it easier to control quality and scale up the culture.The strong glioma-specific tropism of NSC1 cells makes targeting gene delivery possible. *In vitro* migration assays showed that NSC1 and NSC3 cells could migrate specifically toward glioma cell lines, with a tropism similar to that of C17.2, the best-studied NSC cell line. When carrying the suicide gene HSVtk, NSC1 cells demonstrated strong antitumor effects *in vitro* because of their resistance to phospho-GCV. It has been reported that NSCs are more resistant to toxins because of the high level of expression of ATP binding cassette transporter, which makes NSCs more efficient than other cellular vectors in suicide gene therapy. When stem cells are used in therapeutic applications, tumorigenesis is a concern. Thus, the safety and efficacy of this ESC-derived stem cell vector should be further investigated in large-scale preclinical studies.

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